

EFFECT OF ENVIRONMENTAL pH ON THE PROTEOME OF THE DENTAL PATHOGEN *STREPTOCOCCUS MUTANS*

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Bachelor of Science (Honours I)

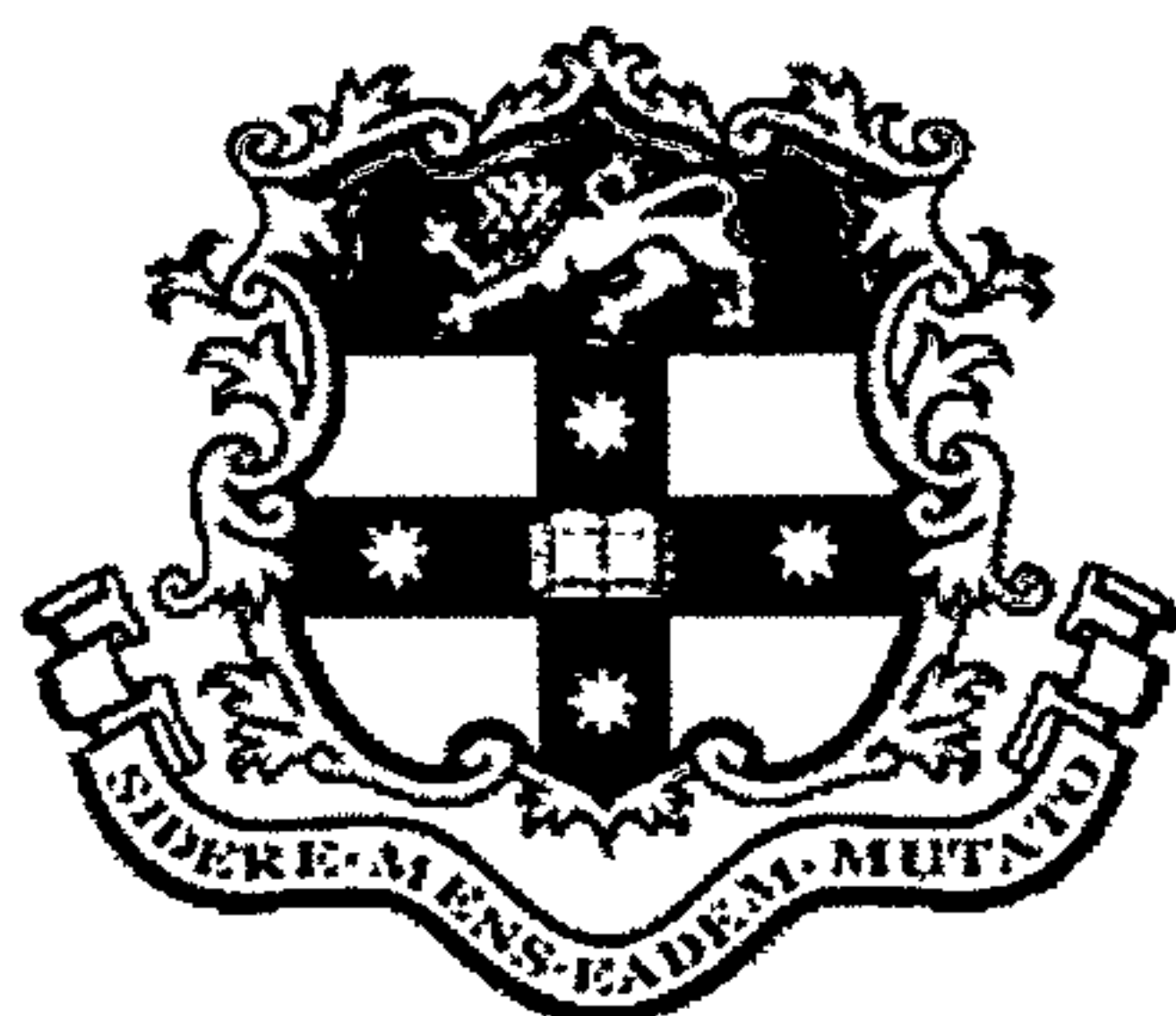
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DECLARATION

The research in this thesis is my own work conducted at the Institute of Dental Research at Westmead Hospital and at the Australian Proteome Analysis Facility at Macquarie University.

This thesis contains original material that has not been submitted for the award of any degree or diploma at any other University or institution. I consent to a copy of this thesis being available in the University library for consultation, loan and photocopy forthwith.

Alice Chen Liang Len, 15th of December 2005

PUBLICATIONS

Len ACL, Cordwell SJ, Harty DWS, Jacques NA. 2003. Cellular and extracellular proteomic analysis of *Streptococcus mutans* grown in a chemostat. *Proteomics* 3: 627-646.

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GENERAL ABBREVIATIONS

Δ pH	Transmembrane pH gradient
2-DGE	two-dimensional polyacrylamide gel electrophoresis
AI	autoinducers
ASB-14	tetradecanoylamido-propyl-dimethyl ammonio-propane sulphonate
ATP	adenosine triphosphate
ATR	acid tolerance response
bp	base pair(s)
BHI	Brain Heart Infusion
BLAST	basic local alignment search tool
CBB	Coomassie Brilliant Blue
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl) dimethyl ammonio]-1-propane sulphonate
CID	Collision induced dissociation
CSP	competence stimulating protein
<i>D</i>	dilution rate
DE	differential expression
DIGE	Differential gel electrophoresis
DMM	Defined Mucin Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dx/dt	change in biomass
ESI	electrospray ionisation
ECC	early childhood caries
<i>F</i>	flow rate
Ftf/s	fructosyltransferase/s
<i>g</i>	gram(s)
<i>g</i>	relative centrifugal force
Gtf/s	glucosyltransferase/s
GCF	gingival crevicular fluid
h	hour(s)
H ⁺	hydrogen ion
HBA	Horse Blood Agar
HPLC	high performance liquid chromatography
IAS	image analysis software
IEF	isoelectric focusing
IPG	immobilized pH gradient
IPS	intracellular polysaccharide
kDa	kilodalton(s)
kV	kilovolt
kVh	kilovolt hour(s)
K _m	Michaelis-Menten constant
LTA	lipoteichoic acid

μ	growth rate
μg	microgram(s)
μL	microlitre(s)
μ_{max}	growth rate maximum
M	molar
M_r	molecular weight
mg	milligram(s)
mL	millilitre(s)
mRNA	messenger ribonucleic acid
MAD	MSS supplemented with 1% (w/v) ASB-14 and 65mM DTT
MALDI	matrix assisted laser desorption/ionization
mg	milligram
mL	milliliter
mM	millimolar
min	minute
M Ω	mega ohms
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSS	multiple surfactant solution
m/z	mass:charge
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
nm	nano meter
ng	nano gram
OM	outer membrane
ORF/s	open reading frame/s
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PEP-PTS	phospho <i>enol</i> -pyruvate:glucose phosphotransferase systems
pI	isoelectric point
PMM/s	peptide mass mapping or maps
PMF/s	peptide mass fingerprinting or fingerprints
pmol	picomole
ppm	parts per million
psi	pounds per square inch
rRNA	ribosomal ribonucleic acid
RPM	revolutions per minute
s	second
S	single limiting substrate
SB 3-10	N-decyl-N, N-dimethyl-3-ammonio-1-propane sulphonate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TAE	Tris acetate-EDTA
TBP	tributyl phosphine
TCA	trichloroacetic acid
TEMED	N, N, N', N' Tetramethylethylenediamine

TOF	time of flight
TIGR	The Institute for Genomic Research
U	Unit(s)
UV	ultra violet
<i>V</i>	culture volume
v/v	volume per volume
W	watt(s)
WHO	World Health Organisation
w/v	weight per volume
<i>Y</i> _{glc}	glucose yield
<i>Y</i> _{ATP}	ATP yield

SUMMARY

Streptococcus mutans is an oral pathogen primarily associated with the initiation of dental caries as the bacterium remains metabolically active when the environment becomes acidic. The mechanisms underlying this ability to survive and proliferate at low pH remain a topic of intense investigation. A global approach to identifying these mechanisms was enabled through the application of the science of proteomics and the sequenced *S. mutans* genome.

S. mutans LT11 was grown at steady state in a chemostat under glucose limitation with a dilution rate of 0.1 h^{-1} at pH 7.0 or pH 5.0 to mimic conditions in a healthy oral cavity in between meal times or that of a carious environment, respectively. Solubilized cellular and extracellular proteins from both pH regimes were analysed using two-dimensional gel electrophoresis (2-DGE). Following trypsin digestion, protein spots were either analysed by matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) or electrospray ionization-tandem mass spectrometry (ESI-MS). The mass spectral data generated was used to search translated contigs of the *S. mutans* UA159 genome sequence database for their corresponding protein sequence. Matched protein sequences were subsequently used to search online protein databases to determine homologous protein identifications.

A baseline map was generated using cellular and extracellular proteins extracted from *S. mutans* cultivated at pH 7.0. A total of 421 protein spots were identified. These corresponded to 200 open reading frames (ORFs) from the translated contigs of the *S. mutans* UA159 genome sequence and thus represented proteins derived from approximately 11% of the total ORFs of this bacterium. Of the proteins identified from both fractions, 170 were cytosolic in nature, one was a cell surface-associated and one was an integral membrane protein. The remaining protein spots had no known function. A comparison of the phenotype of cells grown at pH 5.0 with that of cells grown at pH 7.0, revealed 199 cellular and extracellular protein spots that were differentially or uniquely expressed. Of these, 167 (123 from the cellular fraction) were identified, 70 of which from the cellular fraction were involved with metabolism, particularly glycolysis, alternative acid production and branched-chain amino acid biosynthesis. Proteome

data, in conjunction with end-product and cell yield analyses, were consistent with a phenotypic change that allowed *S. mutans* to proliferate at low pH by expending energy to extrude excess H^+ from the cell, while minimizing the detrimental effects that result from the uncoupling of carbon flux from catabolism and the consequent imbalance in NADH and pyruvate production. The changes in enzyme levels were consistent with a reduction in the formation of the strongest acid, formic acid, which was a consequence of the diversion of pyruvate to both lactate and branched-chain amino acid production when *S. mutans* was cultivated in an acidic environment.

Sixty-one other protein spots (53 from the cellular fraction) were found to be associated with stress-responsive pathways involved in DNA replication, transcription, translation, protein folding and proteolysis. The 61 protein spots represented isoforms or cleavage products of 30 different proteins, of which 25 were either upregulated or uniquely expressed during acid-tolerant growth at pH 5.0. Among the unique and upregulated proteins were five that have not been previously identified as being associated with acid tolerance in *S. mutans* and/or which have not been studied in any detail in oral streptococci. These were the single-stranded DNA-binding protein, Ssb, the transcription elongation factor, GreA, the RNA exonuclease, polyribonucleotide nucleotidyltransferase (PnpA), and two proteinases, the ATP-binding subunit, ClpL, of the Clp family of proteinases and a proteinase encoded by the *pep* gene family with properties similar to the dipeptidase, PepD, of *Lactobacillus helveticus*. The identification of these and other differentially expressed proteins associated with an acid-tolerant-growth phenotype provides new information on targets for mutagenic studies that will allow the future assessment of their physiological significance in the survival and proliferation of *S. mutans* in low pH environments.

Chapter 1 - Introduction¹

The repertoire of proteins expressed within a cell, organ or organism at any one time is known as the proteome of that cell, organ or organism. Many proteome phenotypes can exist throughout the lifetime of a tissue or cell (Wasinger *et al.*, 1995). The completion of genomic sequences of organisms from all three domains, Archea, Prokarya and Eucarya, in conjunction with tools capable of separating and analysing proteins on a micro-scale has enabled the study of proteomes, or the science of proteomics, to become a feasible proposition. In 1995, the parasitic bacterium, *Mycoplasma genitalium*, was the first bacterium to be subjected to proteome analysis using two-dimensional gel electrophoresis 2-DGE coupled with mass spectrometry (MS) to identify the protein spots on the 2-DGE gels (Wasinger *et al.*, 1995; Rabilloud, 2002). Since then many bacterial researchers have realised the potential of proteomics for characterising the protein profiles of bacteria subjected to different environmental stimuli, or to determine differences between strains, or the effect of a mutation on the phenotype of a cell (van Bogelen, 1999; Washburn and Yates, 2000; Cordwell *et al.*, 2001). The use of comparative proteomics to elucidate virulence mechanisms at the molecular level has been at the forefront of many bacterial proteome studies and in many cases has coincided with the completion of the genomic sequence of the bacterium being studied.

The application of comparative proteomics to study *Streptococcus mutans* grown under conditions that mimic those in dental plaque, as described in this thesis, has allowed a comprehensive understanding of two of the most important virulence traits of this organism – aciduricity (acid tolerance) and acidogenicity (continuation of metabolism under low pH). Mutans streptococci are a group of streptococcal species that have been found to be commonly associated with dental caries (Tanzer *et al.*, 2001). By virtue of the fact that members of the mutans streptococci, particularly *S. mutans*, are the most frequently isolated bacteria associated with various forms of carious lesions (Tanzer *et al.*, 2001), it is well accepted that *S. mutans* is a major aetiological agent associated with the initiation of dental caries (Hamada and Slade, 1980; Loesche, 1986). *S. mutans* as well as other saccharolytic oral bacteria ferment exogenous

¹ This chapter was completed in February 2005.

carbohydrates to produce acidic fermentation by-products that lead to enamel demineralisation (Mandel, 1979; Rolla, 1989). Much research using a reductionist approach has been devoted to characterising the regulation of carbohydrate metabolism in *S. mutans*. For example, the glycolytic pathway and alternative fermentation pathways which facilitate survival and continued proliferation under low pH conditions have been intensely investigated (Duncan, 2003; Kuramitsu, 2003). More recently, comparative protein profiles have been used to study the mechanisms that regulate acid adaptation, leading to the observation that multiple simultaneous protein responses are elicited under low pH conditions (Hamilton and Svensäter, 1998; Svensäter *et al.*, 2000).

The manner in which knowledge of the *S. mutans* genomic sequence has enabled the science of proteomics to be applied to the research reported in this thesis and, as a result, has led to a comprehensive understanding of the *S. mutans* phenotype required for acid tolerant growth is reviewed in detail in the following sections, along with aspects of dental caries initiation, particularly the role of host and microbial factors.

1.1 Genomics

1.1.1 Bacterial genome sequencing

Interest in bacterial genetics flourished following the announcement of the discovery of DNA and the determination of its structure by Watson and Crick in the 1950s (Watson and Crick, 1953; Watson and Crick, 1953). This led ultimately to the development of recombinant DNA technologies and its application to earlier research on bacterial genetics. These techniques use a reductionist approach in which individual genes and their products are targeted and analysed, usually through the creation of bacterial mutants. This enables the properties and functions of proteins to be elucidated (Duncan, 2003). Through the availability of sequencing technology, the bacteriophage Φ X174 became the first genome to be completely sequenced in 1982 by Sanger and his colleagues (Sanger *et al.*, 1982). They used a newly developed “shotgun” approach to sequencing, which was based on sequencing and assembling random fragments of the genome.

In 1995, *Haemophilus influenzae* became the first bacterial species to have its genome entirely sequenced using the “shotgun” approach (Fleischmann *et al.*, 1995). Currently (February 2005), 203 bacterial genomes have been sequenced and 624 are in the process of being completely sequenced ([http://www.genomesonline.org/index.cgi?want=Published+Complete+Genomes](http://www.genomesonline.org/index.cgi?want=Published+Complete+Genomes;); <http://www.genomesonline.org/index.cgi?want=Prokaryotic+Ongoing+Genomes>).

Improvements in genome sequencing and annotation in the past decade have resulted in the development of faster sequencing methods. These protocols, coupled with the availability of sophisticated computational algorithms and the reduced overall cost of sequencing have enabled rapid assembly, gene prediction and annotation of entire genomes, that contain less sequencing errors and a higher quality of annotation than previously obtained data (Nelson, 2003). The genomes of the bacterial species that have or are being sequenced fall into three categories. The first category comprises of pathogens and represents the majority of genomes that have or are being sequenced. This group includes *S. mutans*. The second group consists of bacteria that have commercial significance, such as *Corynebacterium glutamicum*, which is used for the industrial production of amino acids for food enhancers. The third group comprise unusual or interesting bacteria, such as *Deinococcus radiodurans*, which is known to be the most radiation-resistant bacterium, as well as ‘model’ organisms, such as *Caulobacter crescentus*, which is used to study the bacterial cell cycle and asymmetrical cell division.

The availability of complete bacterial genome sequences has greatly expanded the collective knowledge of genes already characterised by other means in specific bacterial species and provided a powerful database for obtaining a better understanding of the biology of a given species through analysis of genomic organisation and biological attributes such as virulence, replication and survival mechanisms. Comparison of the genetic makeup between species and strains has led to insights into genome function and evolution. These studies have provided an enhanced understanding of gene arrangements on chromosomes and the conservation and disparities in gene sequences within and between species. They have also allowed the identification of genes that express products that may be used to synthesise vaccines or that are targets for potential antimicrobial therapies, as well as genes that are of commercial importance. In addition, complete bacterial genome sequences also emphasise the number of genes with

unassigned functions. It has been estimated on average that close to half the predicted ORFs of all bacterial genome sequences have unassigned function (Fraser *et al.*, 2000).

While there are many advantages of having a completed genome sequence for a given species, discrepancies have been encountered with the accuracy of the sequences themselves (Schwartz, 2000) as well as the accuracy of the annotation that accompanies the sequencing (Bocs *et al.*, 2002). These inaccuracies can be introduced when genes are not functionally assigned but are given a function based solely on sequence similarity. This error can cascade and lead to further incorrect assignment if another gene is assigned using the previously incorrectly assigned gene (Bork and Bairoch, 1996). Therefore, in order to best assign a function to a gene in the genome of any organism, one needs to examine the gene product(s) directly.

1.1.2 Sequencing the genome of *S. mutans*

Since *S. mutans* is understood to be one of the leading causes of dental caries, sequencing of the genome of *S. mutans* serotype C strain UA159 was undertaken by Ferretti and colleagues at the University of Oklahoma Advanced Center for Genome Technology during the late 1990s (<http://www.genome.ou.edu/smutans.html>). The aim of the genome sequencing project was to provide further insight into the mechanisms that are employed by *S. mutans* to survive and proliferate under the adverse environmental conditions that prevail within the oral cavity, including those that combat host factors and microbial competition in dental plaque (Ajdić *et al.*, 2002).

The genome sequencing used a shotgun approach based on 12,000 individual double stranded templates generated from a randomly sheared library of the *S. mutans* UA159 genome. The templates were cloned into pUC plasmids and sequenced using universal forward and reverse pUC plasmid primers. The closure phase was carried out using custom designed synthetic primers to assemble the sequences and make available a circular contiguous sequence with a high level of accuracy. The complete sequenced and mapped genome was published in October 2002 (Ajdić *et al.*, 2002) and made available on-line by GenBank (Accession no. AEO14133) as well

as the ORALGEN website (<http://www.oralgen.lanl.gov/oralgen/bacteria/smut/>). The genome consists of 2.03 Mbp and contains 1,963 ORFs of which 63% have been assigned a putative function and 37% have no known function (Ajdić *et al.*, 2002). Based on an analysis of this genomic data, several characteristics have been noted. These included the ability of *S. mutans* to metabolise a wider range of sugars than other Gram-positive bacteria. For instance, 15% of the ORFs of *S. mutans* are involved with carbohydrate transport (Ajdić *et al.*, 2002). In addition, *S. mutans* also possesses genes required for the synthesis of all 20 amino acids (Ajdić *et al.*, 2002). More recently, the *S. mutans* genome sequence was compared with 3 other prominent streptococcal species; *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Ferretti *et al.*, 2004). The analysis has provided new insight into how each bacterium has uniquely evolved to evade recognition by the immune system of the host. The comparison has also emphasised the close sequence similarity between the 4 species of streptococci (Ferretti *et al.*, 2004) (Table 1.1).

Table 1.1 A comparison of ORFs between streptococcal species.

Strain	Sequence identity (%)			
	<i>S. pyogenes</i> ^a	<i>S. agalactiae</i> ^b	<i>S. pneumoniae</i> ^c	<i>S. mutans</i> ^d
<i>S. pyogenes</i> ^a	100	77	68	69
<i>S. agalactiae</i> ^b	69	100	67	68
<i>S. pneumoniae</i> ^c	66	72	100	70
<i>S. mutans</i> ^d	65	70	68	100

^a Strain SF370 (type M1); ^b Strain NEM316 (type III); ^c Strain TIGR4 (serotype 4); ^d Strain UA159 (serotype c) (Ferretti *et al.*, 2004).

1.1.3 Functional genomics

Functional genomic investigations usually require a complete genome sequence as a primary tool to drive the generation and analysis of global gene expression. mRNA products collectively expressed from a genome of a cell at any one time are known as its ‘transcriptome’ (Velculescu *et al.*, 1997). In conjunction with the provision of a complete genome sequence, transcriptomics

provides an insight and map of the biological processes of a cell and their functional importance. Transcriptomics is therefore the analysis of the globally expressed set of cellular mRNA transcripts at any point in time (Celis *et al.*, 2000; Harrington *et al.*, 2000; Gros, 2003). All technologies used to investigate mRNA on a genome-wide scale are fundamentally based on the utilisation of high-density microarray DNA chips and in essence involve the total complementary DNA (cDNA) samples derived from the total mRNA transcripts extracted from the cellular sample of interest and hybridised to individual genes or to oligonucleotides representing the genes of the cell immobilized on solid surfaces (Gros, 2003; Liu, 2005). The information obtained by the complementation of available genomic data and the application of microarray technology provides insight into gene regulation. These technologies can also group genes into modules that are potentially co-regulated by specific transcriptional regulators, classify genes into functional categories and associate genes with regulatory pathways. However, the primary importance for investigating the transcriptomes of bacteria is to provide information on genes that can be used as diagnostic targets as well as to understand the regulation of genes involved in virulence. To date, there have been no global microarray studies applied to *S. mutans* as microarray chips have only recently (2004) become available. However, it is clear that investigating the mRNA expression profile of this bacterium should help enhance our understanding of its pathogenic potential.

Monitoring gene expression from a transcript level is one strategy that can be used to investigate the functional genome (Greenbaum *et al.*, 2002). However, even though mRNA is generally needed to produce a protein, the transcriptomic approach does not provide a consistent representation of protein abundance (Anderson and Seilhamer, 1997; Gygi *et al.*, 1999). This limitation is due to the fundamental biological differences between transcriptional and translational products, since transcriptome analyses do not take into consideration the physiological function of proteins. These include, (i) post-transcriptional RNA splicing; (ii) loss of mRNA translational potential due to degradation; (iii) post-translational modifications of proteins (iv) protein-protein or protein-substrate interactions; (v) multi-functional roles of proteins; and (vi) proteolysis. Thus, although the expression of many *S. mutans* virulence traits are controlled at a transcriptional level (For reviews see [Banas, 2004; Lemos *et al.*, 2005]), such knowledge does not provide a complete view of the biological activity or mechanism by which

proteins alter the cellular state. Ideally, combining functional genomic strategies such as transcriptome analysis with the knowledge of the cell's proteome should provide a total holistic analysis of the functional genome of a bacterium. Unfortunately, such studies are currently in their infancy, and many laboratories have therefore directed their attention to studying the proteome of the cell (Hochstrasser *et al.*, 2002; Hegde *et al.*, 2003; Hack, 2004).

1.2 Proteomics

As previously indicated, a proteome encapsulates a 'snapshot' of all proteins simultaneously expressed in a cell, organ, organelle, tissue or organism under a given state, and any organism can possess a number of proteome profiles depending on time, its physiological state or on environmental constraints. It is 10 years since the inception of the term proteome (Wasinger *et al.*, 1995), and since then there has been an explosion of interest in proteome analysis from research groups with a diverse range of biological interests. Proteomics requires four complementary elements; a genomic sequence, protein separation technology, MS and bioinformatics (Wilkins *et al.*, 1997; Blackstock and Weir, 1999) (Fig 1.1). In the pre-proteomics era, traditional protein research was tedious and employed a reductionist strategy. The introduction of proteome technologies has enabled a systematic approach to elucidating the complexity of physiological processes in a comprehensive and high-throughput manner (Hochstrasser, 1998).

1.2.1 Proteome analysis tools

The biological function of an organism is served by a dynamic repertoire of proteins which have been produced through the regulated flow of genomic information to functional protein synthesis. A rapidly expanding catalogue of completed genome sequence data from organisms belonging to all three biological domains is now available. The rate at which new information is becoming available has brought to light the lack of comprehensive proteome data for organisms grown under a variety of physiological conditions.

A method which enabled the display of a set of proteins simultaneously based on their charge

and M_r was developed in the mid 1970s. This separation technology is known as 2-DGE (Klose, 1975; O'Farrell, 1975). Currently 2-DGE involves 'focusing' or separating proteins according to their pI using an immobilised pH gradient (IPG) (see 1.2.3 below), and subsequently separating the focused proteins in a second dimension, based on their M_r , using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (see 1.2.3 below). It was not until the coupling of this technology with highly sensitive MS in conjunction with published genomic sequences that high-throughput proteomics became a reality. New analytical technologies are currently being applied to the proteomics field which promise to deliver better or complementary results to 2-DGE. However, many of these complementary techniques were still in their early stages of trial when the research reported in this thesis was commenced (van den Bergh and Arckens, 2004). The complementation of 2-DGE and MS, now deemed as 'classical' proteomics, is still considered to be the leading method of choice for displaying hundreds or even thousands of proteins simultaneously as a map which can then be selectively or non-selectively analysed in a high-throughput manner (Corthals *et al.*, 2000; Rabilloud, 2002; Gorg *et al.*, 2004) (Fig. 1.1).

1.2.2 Isolation and solubilisation of cellular proteins

The first and most important step in proteome analysis by 2-DGE is solubilizing the proteome and separating it from non-proteinaceous material. Depending on the method chosen, protein extraction will affect how the proteins focus in the first dimension which, in turn, affects the quality and display of proteins in the second dimension. To obtain pure proteins from a biological sample, a method must be devised that incorporates the quantitative extraction of the proteins and frees them from non-proteinaceous substances such as nucleic acids, lipids, carbohydrates and ions, and keeps them solubilised or in a physicochemical stable state in preparation for subsequent 2-DGE analysis (Rabilloud, 1996). In order to achieve this, chemicals are required that solubilise, disaggregate, denature and reduce the proteins (Rabilloud, 1996; Herbert, 1999; Shaw and Riederer, 2003). To this end, the main constituents of proteome extraction buffers are surfactants, chaotropes, reducing agents, and carrier ampholytes. Zwitterionic surfactants are most commonly employed due to the fact they do not alter the charge state of proteins and do not interfere with isoelectric focusing (IEF). 3-[(3-cholamidopropyl)

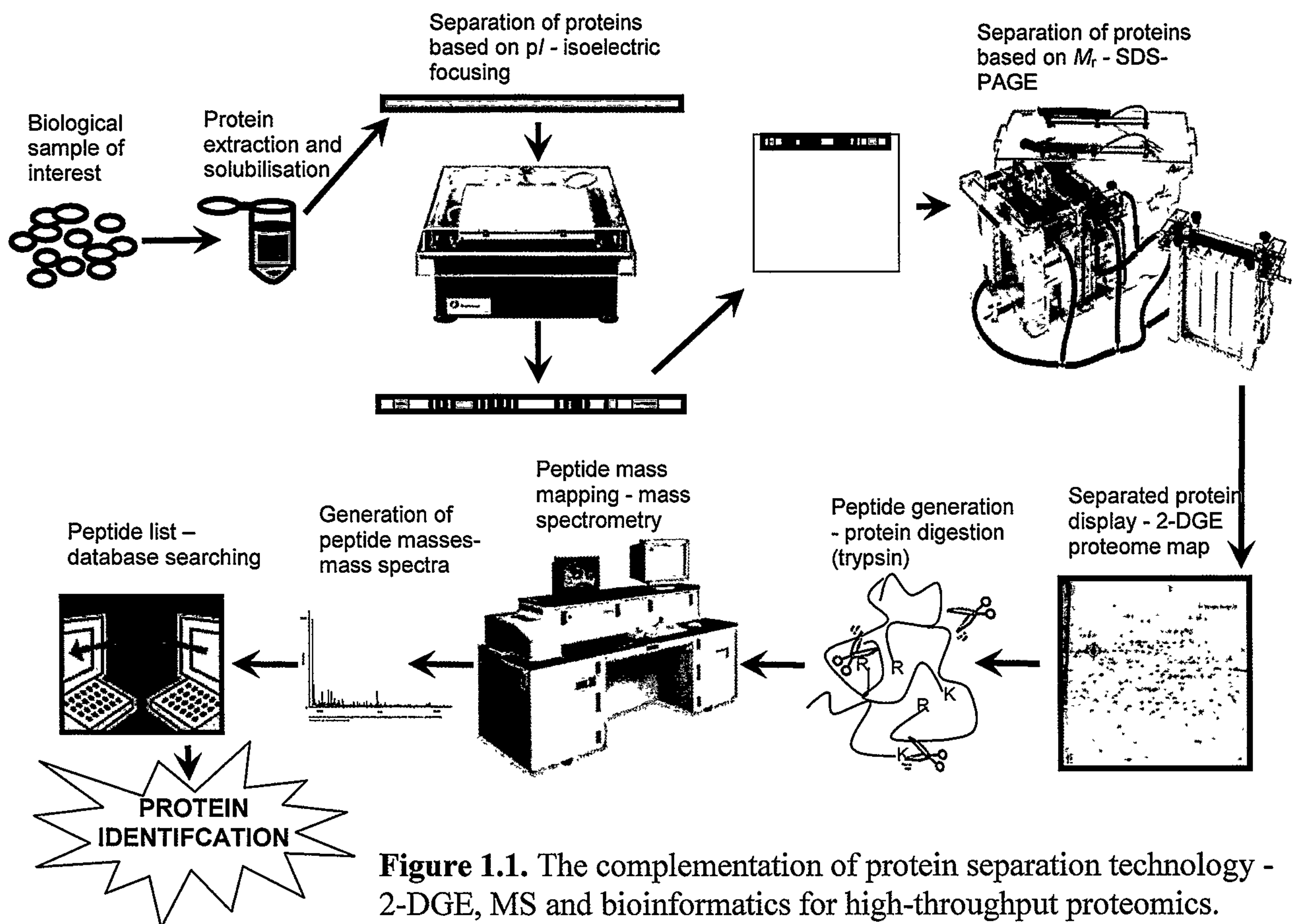


Figure 1.1. The complementation of protein separation technology - 2-DGE, MS and bioinformatics for high-throughput proteomics.

dimethyl ammonio]-1-propane sulphonate (CHAPS) is a highly effective solubilizing agent for proteins and is one of the most commonly used zwitterionic surfactants. Other surfactants include *N*-decyl-*N*, *N*-dimethyl-3-ammonio-1-propane sulphonate (SB 3-10) and tetradecanoylamido-propyl-dimethyl ammonio-propane sulphonate (ASB-14), which are better at solubilizing hydrophobic proteins than CHAPS (Henningsson *et al.*, 2002; Shaw and Riederer, 2003). Chaotropes, like surfactants, denature proteins but their mode of action is to break hydrogen bonds and thus expose the hydrophobic cores of proteins. Both thiourea and urea are commonly used chaotropes and are often used in conjunction with one another. While thiourea itself requires a high ratio of urea to become soluble in H_2O , this combination of chaotropes has been demonstrated to improve the solubility of proteins and increase their resolution in the second SDS-PAGE dimension of 2-DGE (Rabilloud, 1996; Rabilloud *et al.*, 1997; Herbert, 1999). Reducing agents split disulfide bonds and unlike chaotropes and surfactants act to unravel tertiary structure. Common reducing agents are dithiothreitol (DTT) or 2-

mercaptoethanol. More recently, tributylphosphine (TBP) has been used as it has been shown to maintain reducing conditions throughout IEF. Furthermore, as TBP does not contain a free thiol group it does not interfere with the second dimension SDS-PAGE allowing for a single alkylation and reduction step (Herbert *et al.*, 1998). Last, but not least, carrier ampholytes are now commonly added to samples. Carrier ampholytes enhance the focusing of proteins during IEF not only by separating and moving proteins to their respective pI's but also by scavenging cyanate ions and precipitating nucleic acids during the centrifugation steps of the protein solubilizing procedure (Shaw and Riederer, 2003).

1.2.3 2-DGE

High resolution large format 2-DGE allows for the separation of hundreds if not thousands of proteins extracted from a biological sample and displays them in the form of a map for subsequent identification by MS. In principle, 2-DGE involves the separation of proteins in a gel matrix in a consecutive two step process using an electric field based on two independent physiological properties of the proteins. In practice, as noted (1.2.1), the first step, or first dimension IEF, involves the separation of proteins based upon their pI and the second step, or second dimension separation by SDS-PAGE, involves the separation of the proteins based on their M_r .

In the first dimensional separation, proteins migrate to their pI on the IPG strip until they have no net charge and do not move further in the electric field. IPG strips are ultra thin precast polyacrylamide gel strips with immobilised carrier ampholytes that are used to create a pH gradient. Commercial IPG strips vary in length and gradient ranges between pH 2.5 to 12 (Gorg *et al.*, 2000; Gorg *et al.*, 2004). Initially proteins were focused in the first dimension using pH gradients generated in IEF gels using carrier ampholytes. This method was superseded with IPG-based strips, which have proven to be advantageous for 2-DGE as they prevent cathodic drift and lead to an increase in reproducibility, loading capacity, resolution and the separation of proteins with extreme pI's (Bjellqvist *et al.*, 1982; Gorg *et al.*, 2000; Gorg *et al.*, 2004). Limitations, such as the inability to detect low abundance proteins and mediocre resolution due

to protein co-migration that commonly occurs with the use of both broad (~7 pH units) and narrow (~3 pH units) range IPG strips, has recently been largely overcome with the employment of one pH unit 'ultrazoom' IPGs (Gorg *et al.*, 2000; Hoving *et al.*, 2000).

Solubilized and denatured proteins in solution can be applied to the IPG strips in two ways. First, in conjunction with the rehydration of the IPG strip, and secondly, using a cup or paper-bridge with a pre-swollen IPG strip (Gorg *et al.*, 2000). Usually a cup or paper-bridge procedure is only required for basic proteins that are to be separated on basic pH range IPG strips. The use of this procedure significantly decreases the chance of electroendosmosis occurring. Paper-bridge loading is also used when large amounts of protein or a high volume of sample is to be focused (Gorg *et al.*, 2000). Proteins loaded onto IPG strips are separated by IEF on a flat bed horizontal electrophoresis unit. After IEF, the IPG strips which have a stable pH gradient can then be stored indefinitely at -80°C.

To maximise the release of the isoelectrically focused proteins into the second dimension SDS-PAGE, an equilibration step is usually incorporated. Typically, IPG strips are equilibrated by bathing them in a denaturing cocktail solution containing zwitterionic as well as non-zwitterionic detergents, reducing agents and alkylating agents (Gorg *et al.*, 2000). The second dimension SDS-PAGE is typically performed using a vertical slab-gel that enables the separation of isoelectrically focused proteins on the orthogonal axis in respect to their M_r (Walsh and Herbert, 1999). The denatured, negatively charged SDS polypeptides are introduced into the gel, in a sieve like fashion using standard SDS-PAGE conditions (Laemmli, 1970). Large-gel based 2-DGE is preferable, compared with small format 2-DGE, for protein separation as it allows for enhanced protein resolution by decreasing spot overcrowding, thus allowing for subsequent accurate excision of proteins for MS analysis (Nordhoff *et al.*, 2001). Once proteins are separated in the second dimension by SDS-PAGE, the proteins can then be fixed within the gel and stained with appropriate protein staining reagents prior to analysis.

1.2.4 Staining of 2-DGE gels

Protein staining allows the visualisation and imaging of the separated proteins in preparation for

analysis. Several approaches are commonly used for the detection of proteins in polyacrylamide gels (Patton, 2002). For proteomics, the choice of which staining technology to use in analysing the proteome is primarily dependent on stain sensitivity and stain compatibility with MS. In addition, for comparative proteome analysis where measurement of the amount of protein is important, the stain of choice needs to have a broad linear dynamic range (Lopez *et al.*, 2000). Since there are several stains available for proteome analysis only the stains used in the research reported in this thesis are described below.

(i) SYPRO Ruby is a ruthenium-based fluorescence protein stain that can be visualized using UV or blue-light transilluminators that emit an excitation wavelength of 470 nm and detect an emission wavelength of 610 nm (Berggren *et al.*, 1999). It has gained rapid popularity for its compatibility with high-throughput proteomics (Lopez *et al.*, 2000). One of the notable experimental advantages of using SYPRO Ruby is the simple one-step application for staining SDS-PAGE gels (Lopez *et al.*, 2000; Patton, 2002). In comparison to conventional silver staining, SYPRO Ruby staining also has several other advantages (Patton, 2002; White *et al.*, 2004). These include its ability to:

- (a) detect and highlight protein concentrations of as little as 4-8 ng which is slightly more sensitive than silver staining;
- (b) provide a high standard of reproducibility;
- (c) provide low background ‘noise’;
- (d) provide a linear dynamic range of well over three orders of magnitude;
- (e) be compatible with image analysis; and
- (f) be compatible with MS analysis.

According to the manufacturer (Molecular Probes [Invitrogen, California, USA]), SYPRO Ruby can detect a 5% difference in staining intensities with a 95% confidence level. Moreover, SYPRO Ruby has the added advantage of permitting relative protein quantification between gels, the staining of glycoproteins and double staining with organic dyes such as Coomassie brilliant blue (see 11.2.4 [ii] below). In essence, SYPRO Ruby was the first fluorescent dye that allowed for the sensitive detection of proteins on 2-DGE gels, thus giving rise to more comprehensive proteome maps while allowing differentially expressed proteins to be readily quantified.

(ii) Coomassie brilliant blue (CBB) is a dye-anion that is electrostatically attracted through van der Waals forces to the -NH_3^+ functional groups of proteins under slightly acidic conditions. The staining procedure can be completely reversed under the correct pH conditions (Fazekas de st. Groth *et al.*, 1963). CBB based stains are routinely used in laboratories to detect proteins on polyacrylamide gels following electrophoresis. The widespread acceptance of CBB stain is due to several factors including its relative ease of use, low cost and its compatibility with various microchemical analysis tools, such as MS. CBB, however, lacks sensitivity with at least 30-100 ng of protein being required for minimal detection. It also has a far lower linear dynamic range than other stains (Patton, 2002). As mentioned, the stain is compatible with a number of microchemical analysis techniques, such as Edman degradation and MS making it popular as a direct or double stain for protein visualisation after SYPRO Ruby analysis.

1.2.5 Quantitative proteome analysis: Computational image analysis

Computational image analysis is used for conducting quantitative comparisons of proteomes or comparing proteomes from different origins following 2-DGE display. Images of the 2-DGE results which depict the individual protein spots as well as indicating their amount and characteristics are a prerequisite for image analysis and can be displayed *in silico* using image analysis software (IAS), several of which are now available (Monteoliva and Albar, 2004). Typically, standard IAS algorithms enable spot detection and the matching or overlaying of gel images, thus allowing for protein spot matching from two or more 2-DGE gels. The relative expression of a given pair of corresponding protein spots can then be compared to provide statistical data. Quantification and comparative analysis of proteome phenotypes allows one to decipher which proteins or pathways are involved in a given response.

At the commencement of the research reported in this thesis, z3 software supplied by Compugen (Tel Aviv, Israel) was one such software package that offered the capacity to perform raw-image-based computation and region-based matching, as well as quantification of fluorescently stained protein spots on 2-DGE gels (Smilansky, 2001). Its capacity to reduce the detection of false

positive protein spots, and thus limit the requirement for user intervention, was far superior to other image software available at the time (Raman *et al.*, 2002). For instance, co-migration of proteins is not an uncommon occurrence in 2-DGE (Pietrogrande *et al.*, 2003), but the z3 program is capable of detecting protein spot overlapping. It is, however, limited in its inability to detect a low abundant protein spot obscured by a highly abundant protein spot. The problem of protein spot overlapping can be minimised by separating the proteins in the first dimension using a series of single pH unit IPG strips. If overlapping does occur and a distinguishable separation can not be achieved, the area with the overlapping spots can sometimes be judiciously excised and processed by MS and identified.

1.2.6 Protein analysis and identification: MS and bioinformatics

Following 2-DGE, protein staining and image acquisition, protein spots are excised from the gel either manually, or automatically with a robot, and then washed to remove staining agents such as CBB (Traini *et al.*, 1998; Nordhoff *et al.*, 2001). The proteins are then cleaved *in situ* in the gel plugs either chemically, using cyanogen bromide, or enzymatically using trypsin or some other proteinase (Ward *et al.*, 1990). Trypsin specifically cleaves peptide bonds on the carboxyl side of arginine and lysine (the exception being arginine-prolyl or lysine-prolyl bonds due to steric hindrance) (Smith and Wheeler, 1997), to produce a unique set of peptides that are characteristic of the protein in question. The proteolytic cleavage products are then extracted from the gel plug and analysed by MS. In some instances, it is advantageous to first purify and concentrate the peptides. This is achieved by the use of nano-scale reversed-phase columns and leads to an increase in the sensitivity of the MS analysis which in turn increases the peptide sequence coverage and the confidence with which the protein can be identified (Gobom *et al.*, 1999). Micro-purification columns can be tailor made in-house or are commercially available.

Following protein digestion, the released peptides can be analysed by MS. Fundamentally, a mass spectrometer is an instrument that measures the mass of ionized analytes in a gas phase. It is composed of three essential components; an ion source, a mass analyser and a detector. Ions can be formed in different ways (see below), while the mass analyser separates ions based on their mass-to-charge (m/z) ratio using an electric or magnetic field. As its name implies, the

detector identifies the ions which have passed through the mass analyser and registers the ions at each m/z value (Simpson, 2003).

The first experiments on MS were carried out by J J Thomson in the late 19th century (Thomson, 1899). By the mid 1980s, MALDI - TOF MS was used to analyse protein masses (Fig 1.2A) (Karas and Hillenkamp, 1988; Tanaka *et al.*, 1988). An increase in the sensitivity and accuracy of MALDI-TOF MS instrumentation made it possible to generate peptide mass maps (PMMs) or peptide mass fingerprints (PMFs) from fragmented proteins for protein identification using reflectron MALDI-TOF MS (Fig 1.2B) (Henzel *et al.*, 1993). MALDI-TOF is now considered an indispensable and robust routine tool for proteome analysis (Smith, 2002b; Thiede *et al.*, 2005).

PMM or PMF using MALDI-TOF MS is frequently used as the first strategy for protein identification (Cleveland *et al.*, 1977; Bloch *et al.*, 1980; Phillips *et al.*, 1980; Karas and Hillenkamp, 1988; Henzel *et al.*, 2003; Thiede *et al.*, 2005). PMM is a technique that identifies proteins by matching their enzymatically or chemically fragmented masses (peptide masses) to the theoretical masses generated *in silico*, using the respective chemical or enzyme, from a protein database (Jonsson, 2001).

To perform MALDI-TOF MS, the peptides derived from a protein are spotted onto a MALDI target plate and mixed with a peptide amenable wavelength absorbing matrix, such as α -cyano-4-hydroxy-cinnamic acid, and dried to form a co-crystallised mixture. Pulsed UV light from a nitrogen laser is targeted at the co-crystallised structures on the target plate resulting in ionized peptides being released from the MALDI target plate along with matrix ions and other neutral species. A high potential electric field enables the rapid channelling of mainly singly charged ions ($[M+H]^+$ or $[M-H]^-$) from the ion source into the mass analyser of the mass spectrometer, which are subsequently reflected and detected (Beavis and Chait, 1989; Beavis and Chait, 1989; Beavis and Chait, 1989; Cohen and Chait, 1996; Kussmann *et al.*, 1997; Kussmann and Roepstorff, 2000) (Fig 1.2).

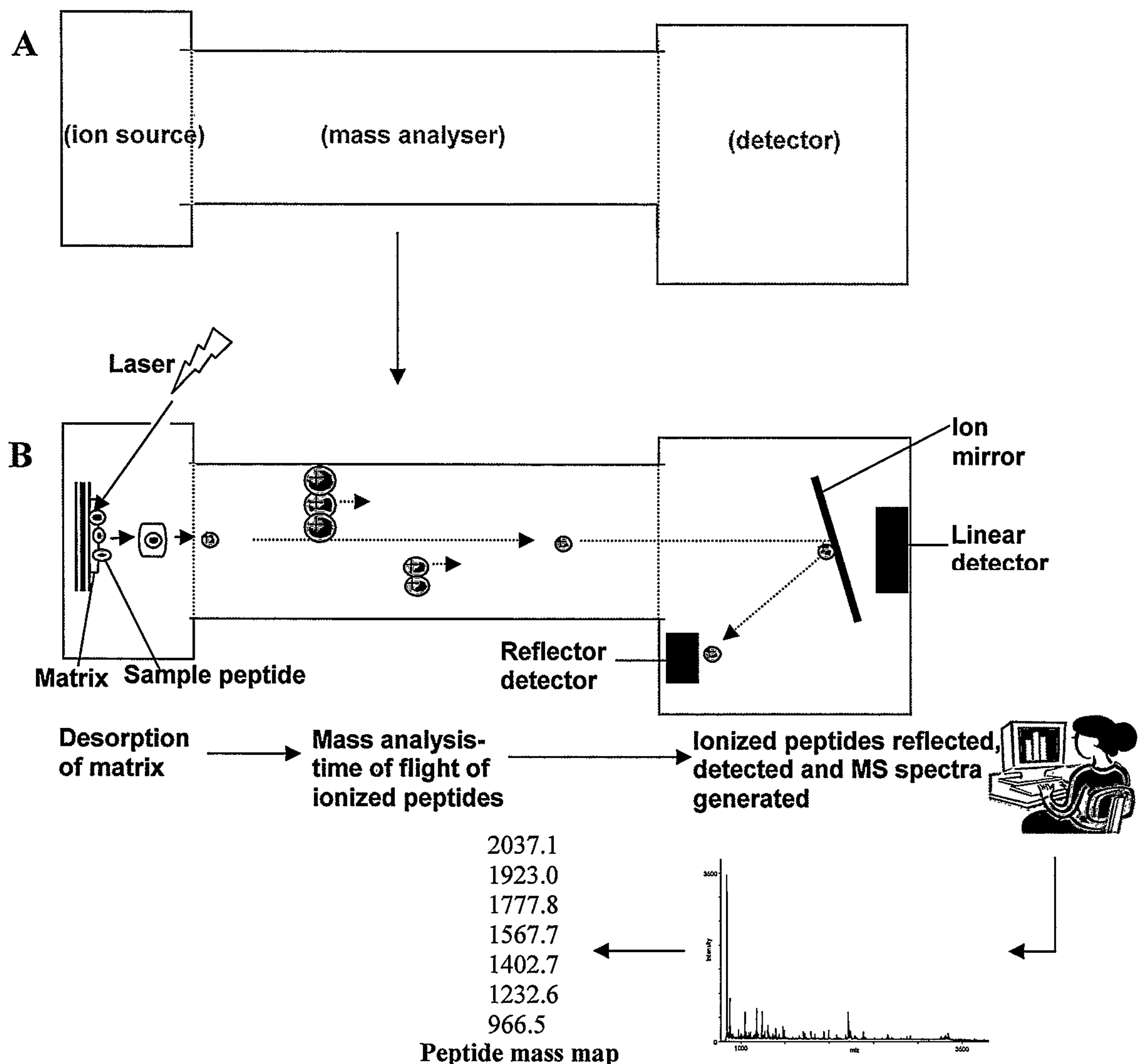


Figure 1.2. Schematic of (A) MALDI-TOF MS and (B) the process of peptide mass analysis by reflectron MALDI-TOF MS and PMM generation.

In some instances it is necessary to sequence peptides to identify a protein of interest since peptide sequence information is more advantageous than PMM data alone for proteins that have not been identified and catalogued in a protein database, or have not been translated from the sequenced genome of an organism. In addition, when the PMM data contains only one or two peptide masses, which can occur with low M_r proteins (< 25 kDa) and/or proteins of low concentration, a confident identification of the protein may not be possible. Until recently,

Edman degradation has been the method of choice for sequencing unknown proteins or peptides (Edman, 1950). The method provides relatively unambiguous sequence data for 4-10 *N*-terminal amino acids (Wilkins *et al.*, 1996). However, a large amount of sample is required for analysis (1-10 pmol) and the method is dependent on the *N*-terminus of the protein not being modified or blocked (Brown and Roberts, 1976; Brown, 1979). In addition, standard sequencing costs are expensive and require the protein of interest to be purified (Henzel *et al.*, 2003). Such limitations are overcome by peptide sequencing using tandem mass spectrometry (MS/MS). This technique is superior in sensitivity and accuracy than Edman degradation. Most importantly MS/MS is a high throughput protein analysis and identification method that is not limited to sequencing from the *N*-terminus of a protein (see below). PMM by MALDI-TOF MS and sequencing by MS/MS are now the two most common methods for rapidly identifying proteins separated by 2-DGE.

De novo sequencing by tandem MS of peptides provides sequence information about the peptide, which is often sufficient for protein identification. This differs from PMM data which only provides a single attribute; unambiguous M_r s of peptides derived from the protein. Peptides for tandem MS are obtained in the same manner as in MALDI analysis. However, the subsequent analysis via MS/MS consists of several mass selection events. First, like MALDI, individual peptide masses are determined from the fragmented protein. One of the analysed peptides is then selected and designated the parent (precursor) ion and further fragmented. This subsequent fragmentation is usually achieved through collision induced dissociation (CID) (Fig 1.3) (Hayes and Gross, 1990). CID is a process whereby an ion and a neutral species collide, resulting, in part, in the conversion of translational energy into internal energy that results in the subsequent fragmentation of the ion (Shukla and Futrell, 2000). The breakdown of the peptide follows a 'ladder' fragmentation pathway and the m/z value of the resulting breakdown ions or daughter (product) ions are then measured in a second MS that produces a series of ions which define the sequence of the 'parent' peptide (Fig 1.3) (Hunt *et al.*, 1986; Biemann, 1990). In order to be detected, daughter ions must carry at least one charge. During ionization of a peptide six kinds of ions are formed and the type of ion produced is dependent on where the bonds in the peptide break. If the charge is retained on the *N*-terminus of a fragment peptide, the ion is either classed as a, b or c. If the charge is retained on the *C*-terminus of a fragment peptide, the ion is either classed as x, y or z. The two main types of ions or complementary ions analysed from peptide

fragmentation are the b- and y-ion series (Roepstorff and Fohlman, 1984). As noted, extensive sequence information can be acquired by tandem MS that spans the whole length of the protein rather than the restriction imposed by *N*-terminal sequencing using Edman degradation.

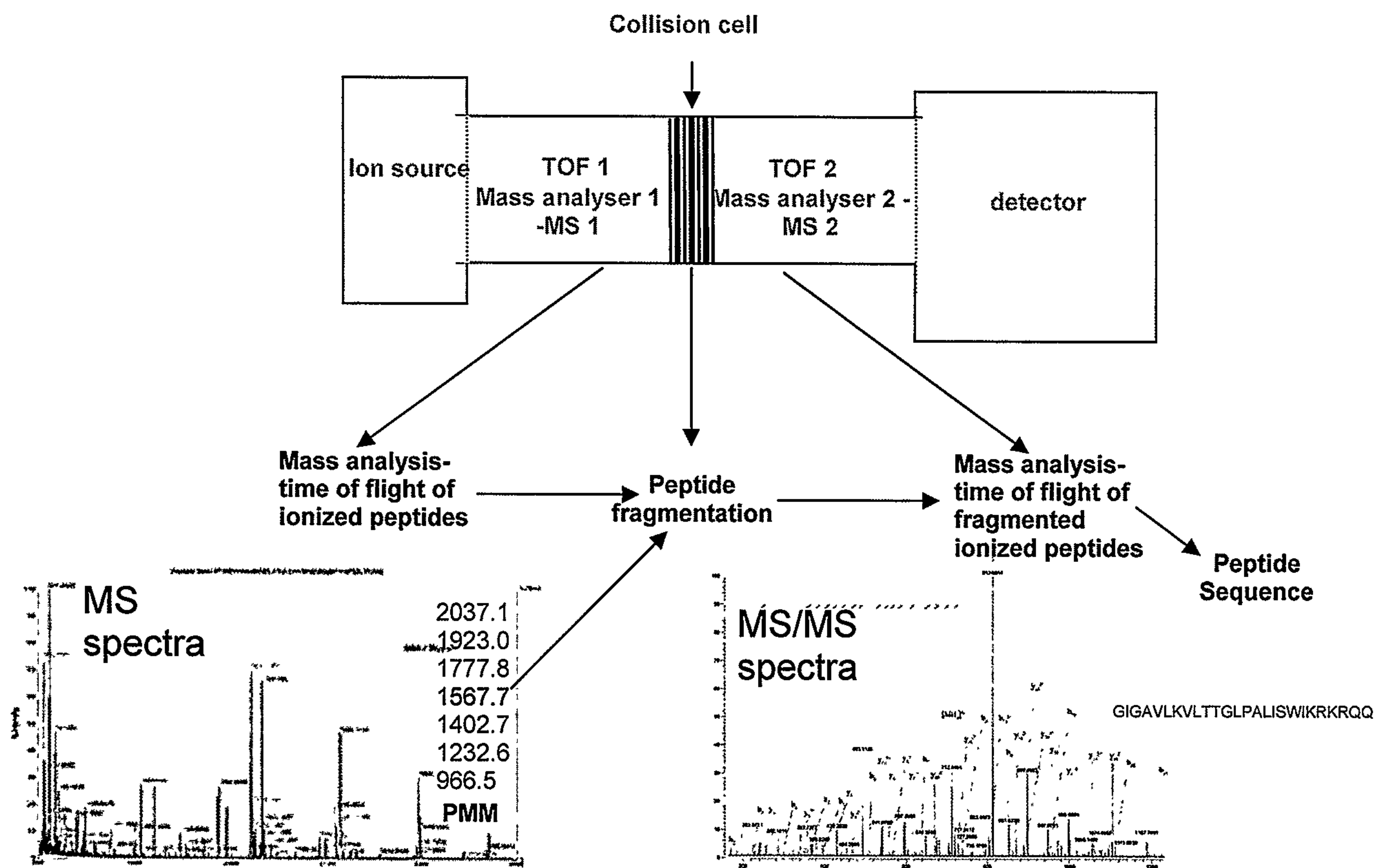


Figure 1.3. Peptide sequencing using tandem MS/MS (see 1.2.6)

1.2.7 Databases- Protein identification and characterisation

In essence, there are two approaches that can be taken to identify MS analysed proteins. These are protein database searches and peptide sequencing. PMM generated data is used to search protein databases. Internet accessible algorithms that cater for multi-species protein database searching include MASCOT (Perkins *et al.*, 1999) and PeptIdent (which is now superseded by more powerful program Aldente [<http://au.expasy.org/tools/aldente/>]), to name but two. Software, such as ProteinLynx (Micromass [Waters, Massachusetts, USA]), which is compatible with MS instrumentation, can interpret raw MS data to provide PMM data. These data can then

be used to search on-line as well as off-line. Off-line queries are advantageous when in-house databases of the species of interest have more information than on-line sources.

To obtain a confident match for PMM data it is advantageous to have a comprehensive database of theoretically digested proteins from the organism of interest. While it is possible to conduct cross species database interrogation with PMM data, generally only conserved proteins can be reliably matched (Wilkins and Williams, 1997). In addition, it is imperative that the sequences used for theoretical digestion are not erroneous, single amino acid substitutions can significantly change the mass of a peptide. Consequently, in order to obtain a confident match when identifying a protein from PMM data, a series of parameters must be set. These include:

- (i) peptide mass tolerance of less than 100 ppm;
- (ii) sequence coverage of greater than 20%;
- (iii) at least four matching peptides; and
- (iv) some comparative estimate of the pI and M_r from the 2-DGE display should also be considered.

A preferred alternate protein identification strategy is peptide sequence analysis using MS/MS or *N*-terminal sequencing as described above (see 1.2.6). This method of protein identification is advantageous as an amino acid sequence of one or more peptide fragments from a protein of interest can be obtained. Typically a peptide sequence of 10 amino acids can be used to search a protein database and retrieve confident protein identification using Basic Local Alignment Search Tool (BLAST), which caters for sequence comparisons (Altschul and Koonin, 1998; Jones and Swindells, 2002).

Completed and published, as well as ongoing microbial genome sequencing projects, can be viewed at Internet sites such as the Genomes OnLine Database (GOLD™ [<http://www.genomesonline.org/>]) (Kyrpides, 1999; Bernal *et al.*, 2001), The Institute For Genomic Research (TIGR [<http://www.tigr.org/>]) or Microbial Genomics ([http:// genome.jgi-psf.org/mic_home.html](http://genome.jgi-psf.org/mic_home.html)). Unfinished microbial genome databases provide comprehensive information on sequencing status, the institutions conducting the sequencing, the anticipated completion date and in some instances a file that contains contigs of the genome that have been

sequenced to date. This data can be useful in protein identification using MS techniques. For instance, when the research for this thesis commenced in early 2000, the *S. mutans* UA159 sequencing project was still in progress, but contigs were readily available to download (<ftp://ftp.genome.ou.edu/pub/smutans/>). Downloaded contigs were translated into six frames using the Flip6Frames program ([Brossard, 1997]; <http://megasun.bch.umontreal.ca/ogmp/ogmpid.html>) accessed through the Australian Genomic Information Service (<http://www.angis.org.au/> WebANGIS) and digested *in silico* with the proteinase, trypsin, used to generate the PMM data of proteins separated from 2-DGE analysis. This created a database that could be used for searching against PMM data.

1.3 Continuous culture

A culture grown under continuous culture in a chemostat is also known as a continuous-flow open culture system (Fig 1.4). A chemostat consists of a homogenously distributed culture where the flow of medium is at a constant rate and a limiting nutrient is immediately metabolised. The outflow of culture is at the same rate as the inflow of medium so that volume of the culture is kept constant (Pirt, 1975). The purpose of continuous culture is to reach steady state where the increase in biomass over an infinitely small time interval is equal to zero and where the increase in cell numbers equals the decrease due to the depletion of the culture volume by inflowing medium, (Monod, 1949). In this system, the dilution rate, D , is defined by the flow rate of the medium into the chemostat relative to culture volume, such that:

$$D = F/V$$

where F is the flow rate and V is the culture volume.

The change in biomass, x , in the growth vessel is given by (dx/dt) . Change in biomass is dependent on the growth rate, μ , and the subsequent dilution of the culture vessel by inflowing medium provided that there is a single limiting substrate, S . Thus:

$$dx/dt = \mu x - Dx.$$

Steady state is established when $dx/dt = 0$ and the change in biomass is constant. Thus:

$$\mu x - Dx = 0 \text{ or } \mu = D$$

As a consequence, D can be manipulated during steady state to alter the growth rate provided that the growth rate is below the maximum growth rate, $\mu_{\max}$. If D exceeds, $\mu_{\max}$, the culture will wash out from the vessel.

The relationship between the growth rate, μ , and limiting substrate concentration at steady state was shown by Monod (1942) and later by Harder and Kuenen (1977) to be:

$$\mu = \mu_{\max}(S/(k_S + S)) = \ln 2 \ t_d^{-1}$$

where k_S is the half rate saturation constant (ie where S is equal to k_S when the growth rate is at $0.5 \ \mu_{\max}$), and t_d is the doubling time of the cells.

Since $\mu = D$ at steady state, then:

$$D = \ln 2 \ t_d^{-1} \text{ or } t_d = D^{-1} (\ln 2)$$

Continuous culture has an advantage over batch culture, or a plug-flow closed culture system, since:

- (i) Both the growth rate and the biomass of the organism of interest can be controlled. This includes fixing the growth rate so that the environment can be altered allowing one to note the differences in effect between growth rate change and environment change (Pirt, 1975). This is not possible using conventional batch culture.

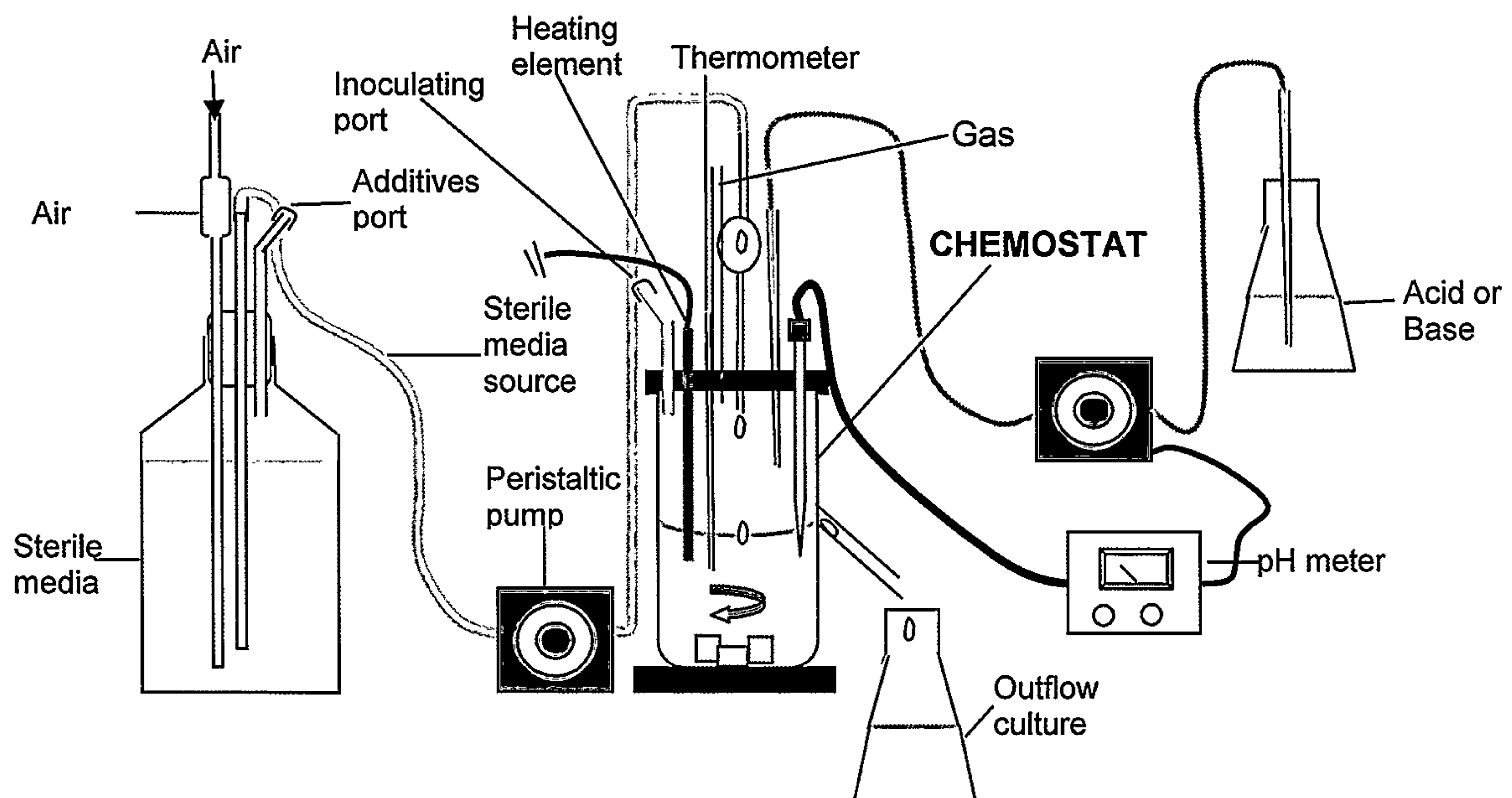


Figure 1.4 Diagram of a chemostat.

- (ii) Substrate-limited growth can be maintained with a constant growth rate, whereas in batch culture, substrate-limited growth is only transient. In batch culture exhaustion of the substrate is accompanied by a change in growth rate (Pirt, 1975).
- (iii) Cells grow continuously and are not subjected to different growth phases as in batch culture where several different growth phases occur (ie lag, exponential and stationary phases) and where each growth phase may express a different protein phenotype. The expression of different proteins during various phases in batch culture poses a problem when attempting to obtain a true phenotypic representation of the whole culture. This problem is not encountered at steady state in continuous culture in a chemostat where a constant growth and physiological state can be selected and maintained.

Chemostats have been used to investigate physiological and growth attributes of various strains of *S. mutans* under conditions that simulate those in the oral cavity, such as alterations in pH (Marsh, 1991; Dashper and Reynolds, 1992; Iwami *et al.*, 1992; Quivey *et al.*, 1995; Jayaraman *et al.*, 1997; Quivey *et al.*, 2000; Crowley *et al.*, 2004), and the effect of pH on chemostat-based biofilms (Li and Bowden, 1994; Li *et al.*, 2001; McNeill and Hamilton, 2003; Welin *et al.*, 2003).

; McNeill and Hamilton, 2004), to name but two. Continuous cultures have also been employed to investigate secreted products particularly metabolic end-products of oral streptococci. For global comparative protein expression analysis one requires reproducible and meaningful data, which in the case of bacteria, can be delivered by the application of continuous culture technology. In fact, chemostat culturing was recently recommended by the Burne laboratory as a tool necessary to prepare comparable cells without growth discrepancies (Lemos *et al.*, 2005). They commented that continuous culture growth in a chemostat was the “gold standard” for comparative physiological or environmental studies. It is for this reason that *S. mutans* was grown at steady state in a chemostat for the research reported in this thesis and the cells used for comparative proteome analysis.

1.4 Dental Caries

While teeth are primarily used for breaking down food and contribute to fluency of speech they also provide a surface for the formation of a dental biofilm known as dental plaque (see 1.4.2.3 below). Teeth are resilient bone-like structures and each tooth is essentially composed of four tissue types - pulp, dentine, cementum and enamel (Fig 1.5 B, D). Enamel protects and envelopes the various underlying tissue types that compose the tooth and therefore is the tissue that is exposed to the environment of the oral cavity. In the case of dental caries, tooth enamel is the primary host tissue that is directly attacked and subsequently demineralised by organic acids secreted as metabolic end products when oral microbial inhabitants accumulate as dental plaque on the tooth surface (Fig 1.5 A, C).

Dental caries is the leading cause of progressive localised tooth destruction in man. In early 2000, the World Health Organisation estimated that 5 billion people worldwide had experienced dental caries (WHO, 24 February 2004, Geneva). Due to its high rate of morbidity, treatment of dental caries places a significant monetary burden on healthcare systems in Western societies. In the United States alone, it was estimated that a total of \$60.2 billion was spent in 2000 on dental services directly or indirectly as a result of dental caries (Allukian, 2000). Unfortunately, in several Asian and Latin American countries, where dental caries is the most prevalent oral disease, healthcare support for treatment of this disease is well beyond the countries financial

resources (WHO, 24 February 2004, Geneva).

Dental caries in toddlers and preschool children is known as 'early childhood caries' (ECC). It is clearly understood that ECC is a public health problem worldwide which can seriously affect the quality of a child's life. At present in the USA, up to 72% of native North American children suffer from ECC (Berkowitz, 2003). In Seoul, Korea, ECC was found to be present in 56.5% of children aged 6-59 months (Jin *et al.*, 2003) while in the Northern Philippines up to 94% of children suffer from ECC (Carino *et al.*, 2003). Children living in under-privileged or developing countries still receive minimal or no education on dental health and public healthcare support for the prevention and treatment of dental caries in many developed nations still remains a neglected health problem. In the USA, 78% of adolescents aged 17 and 98% of 40-44 year olds have or have had tooth decay (Allukian, 2000). A prediction by Reinhardt and Douglass in 1989 calculated that from 1990 to 2030 there would be an increase of 73% in 45 year olds and older and 104% in 65 year olds and older needing restorative dentistry. This equates to a need for 150 and 192 million hours of dental restorative treatment in 1990 and 2030, respectively, in the USA (Reinhardt and Douglass, 1989). Suffice is to say that dental caries affects a wide range of individuals, from children to adults, regardless of socio-cultural or economic backgrounds.

Dental caries is an infectious disease resulting in the demineralisation of tooth enamel due to the fermentation of exogenous or endogenous carbohydrates by bacteria (Fig 1.6). Saccharolytic oral commensal bacteria metabolise sugars, including polysaccharides and breakdown products of glycolipids that become available in their immediate surroundings, or are provided by the host from the degradation of glycoproteins, and ferment them to acidic end-products. The metabolism and release of acidic end-products of carbohydrate metabolism lead to an accumulation of organic acids in dental plaque. The accumulation of the organic acids, in particular lactic acid, shifts the demineralisation/remineralisation equilibrium of tooth enamel towards demineralisation, thus initiating the process of dental caries (Robinson *et al.*, 2000; Aoba, 2004).

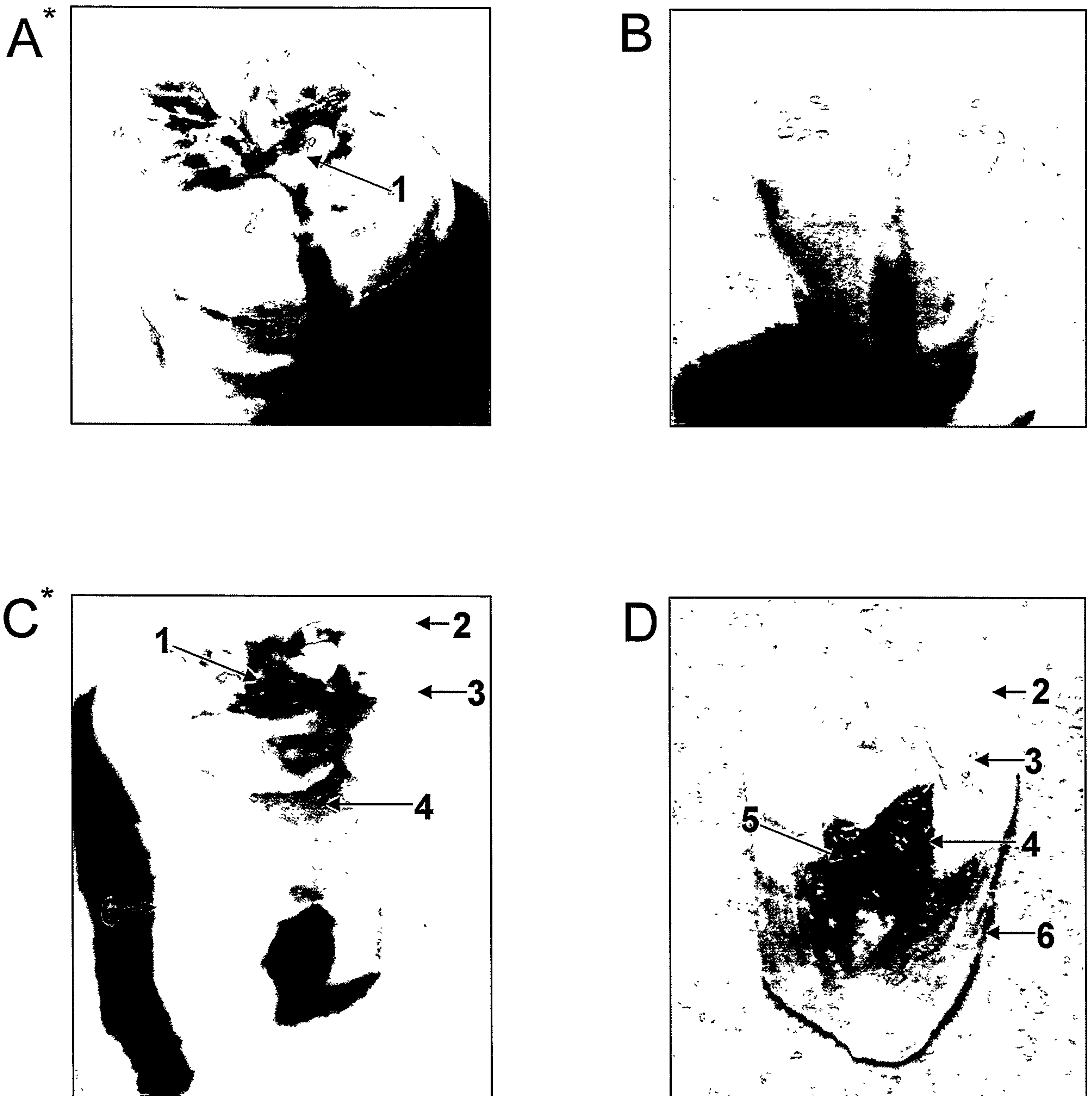


Figure 1.5. Carious (A, C [cross section]) and non-carious teeth (B, D [cross section]). (1) carious lesion, (2) tooth enamel, (3) dentine, (4) pulp chamber, (5) pulp, and (6) cementum.

* Figures A and C courtesy of Dr Ky-anh Nguyen, Institute of Dental Research. Wentworthville, NSW, Australia.

The first signs of dental caries are described as a “white spot” or incipient caries lesion forming on a tooth surface. This is caused by the acid demineralisation process outweighing the natural remineralisation of enamel by saliva. The continuation of localised demineralisation of tooth enamel leads to the destruction of enamel and to the formation of a lesion. If not treated, the caries process will involve the dentine and pulp tissue and even migrate through the apex of the tooth to the surrounding alveolar bone (Fig 1.5 A, C). The induction of the disease is a multi-factorial process, while the severity of the disease is dependant on the dynamic interrelationship of several factors that promote its formation and continuation.

In the 1960s Keyes and Jordan (Keyes and Jordan, 1963) proposed their dental caries theory, which is applicable to all forms of the disease. Their theory is simply and effectively outlined by a Venn diagram (Fig 1.7). It simply states that the three general factors, diet, host and bacteria, influence the formation and advancement of a carious lesion and without all three working in unison dental caries does not occur. However, it has been suggested that a lack of dental hygiene and time should also be equally weighted with the three other factors (Mascarenhas, 1998).

1.4.1 History and Epidemiology of dental caries

Universal paleontological studies on teeth from human remains provide historical evidence that dental caries prevalence increased during the Neolithic period when humans replaced hunting with agriculture as the main source of food. Prior to this period, the estimated caries incidence was 1% in comparison with 5% for agricultural based civilisations (Clarkson, 1999; Ettinger, 1999) . The dental disease that mostly afflicted human groups before the introduction of an agricultural diet was periodontitis (inflammation of the gums) and erosion of tooth surfaces due to attrition (White-Graves and Schiller, 1986). Clay tablet evidence dating back to 4000 BC describes a ‘tooth worm’ theory for dental caries. This widely accepted belief suggested that a demonic worm was the cause of carious teeth and fumigation using burning henbane seed was prescribed to rid or cure the sufferer of the so called “worm” (Levine, 1977; Mandel, 1983).

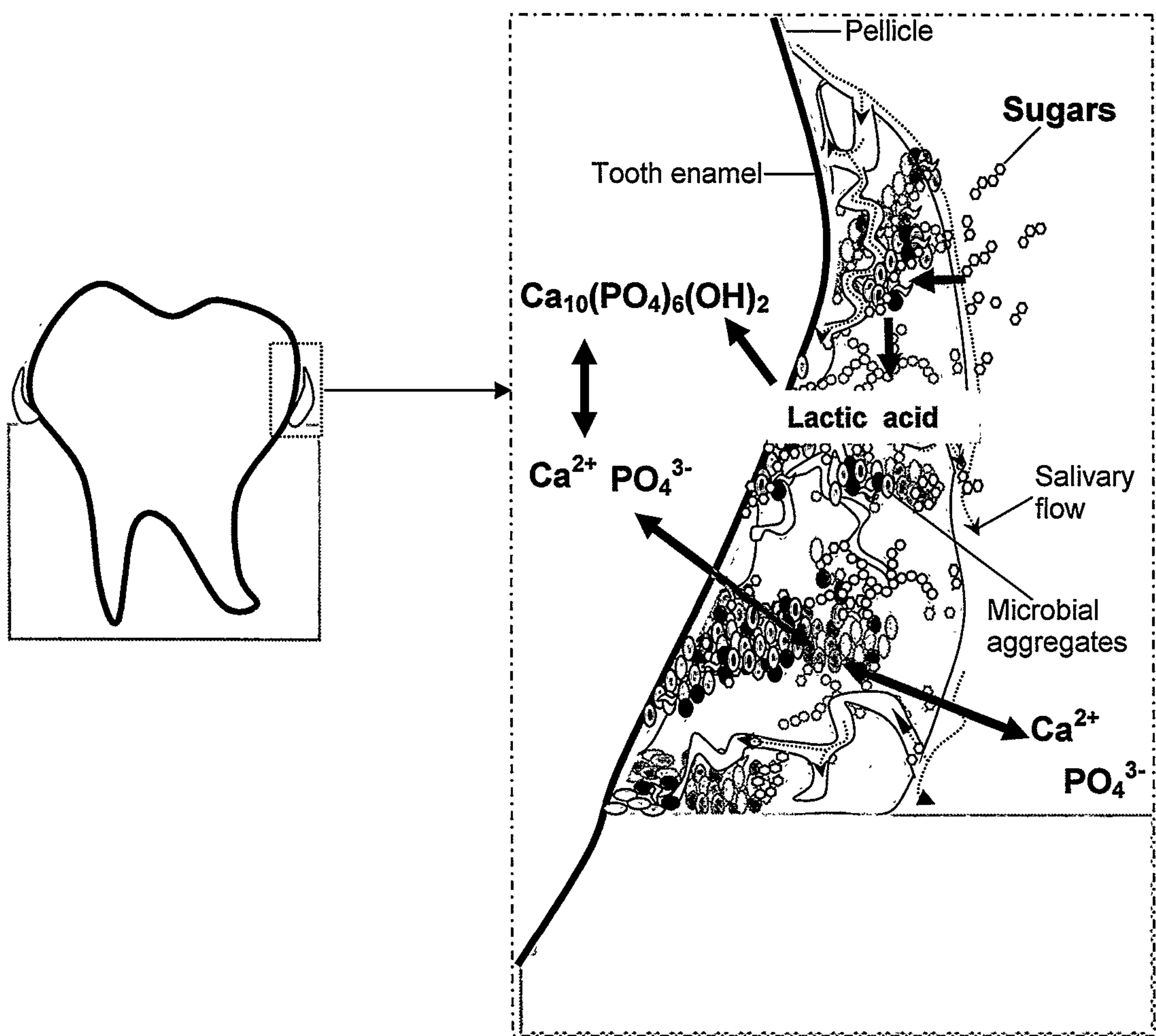


Figure 1.6. The process of tooth demineralisation by lactic acid produced by plaque bacterial carbohydrate metabolism.

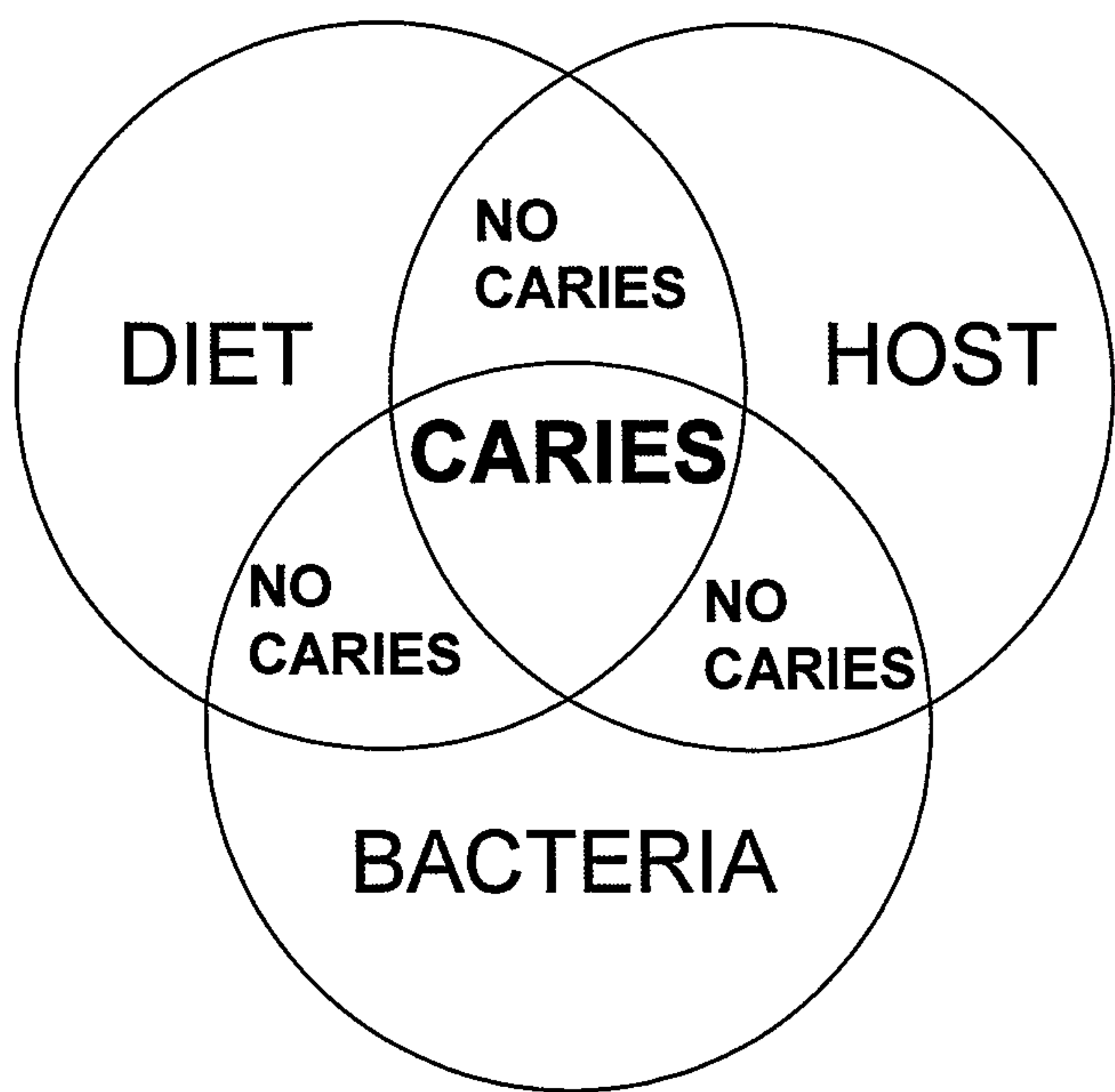


Figure 1.7. Venn diagram illustrating the dental caries theory described by Keyes and Jordan (Keyes and Jordan, 1963). It shows the three factors, diet, host and bacteria, that are needed to promote the formation and advancement of dental caries.

In 1846 in Britain, the repeal of the Corn Laws, which had governed the importation of wheat and sugar prior to this date, saw the prices of both consumables decline. By the turn of the 19th century consumption of these commodities quadrupled, especially by individuals from lower socio-economic backgrounds. A study conducted by Corbett and Moore of the dentition of skulls prior to and after the repeal of the Corn Laws strongly suggests that there was a positive correlation between the increased consumption of refined carbohydrates and the increased prevalence of dental caries (Corbett and Moore, 1976). Late in the 18th Century, John Hunter speculated on the cause of dental decay, but it was not until W D Miller in 1890 first documented dental caries aetiology, that the famous 'Chemico-Parasitic Theory' was born. His theory suggested that oral bacteria converted dietary carbohydrates into acid, which solubilised tooth enamel, leading to the production of a carious lesion (Miller, 1890; Cohen, 1982). In 1924 Clarke isolated an organism from a carious lesion, which was later named *S. mutans* (Clarke, 1924). Proof that *S. mutans* was directly involved in dental caries was confirmed when gnotobiotic rats provided solely with a high sucrose diet did not develop dental caries, while rats infected with *S. mutans* and fed a high sucrose diet formed carious lesions (Orland *et al.*, 1955). By the 1950s dental caries had become pandemic with 90-95% of the population of the developed world being infected (Clarkson, 1999).

1.4.2 Factors affecting dental plaque and caries formation

1.4.2.1 Host factors

The human oral cavity is physiologically and ecologically distinct to other surfaces of the body and is subjected to considerable ecological change from infancy to adulthood. In an infant the dominating surface is the mucosal epithelium such as the tongue, gums and cheeks that are bathed in saliva, which is a complex liquid that mainly consists of water (99%), electrolytes, enzymes, mucins, immunoglobulins, lipids and non-immunoglobulin proteins. As an infant enters early childhood, the eruption of the primary dentition introduces non-shedding surfaces into the oral cavity. The acquired enamel pellicle, otherwise simply known as the pellicle, is a microbially free film that forms on oral hard and soft tissues from constituents in saliva (Hamada and Slade, 1980; Smith *et al.*, 1993; Pearce *et al.*, 1995). It forms by selectively adsorbing

glycoproteins and proteins of host and bacterial origin and aids in selectively facilitating the adhesion of bacteria onto the tooth surface (Hannig *et al.*, 2005). The role of the acquired pellicle in dental caries remains poorly understood (Hay, 1995).

In the oral cavity, saliva also serves a number of other roles including lubrication, the initiation of the digestion of food, and protection of the dentition from enamel decay (Leone and Oppenheim, 2001; Lingstrom and Moynihan, 2003)). The lifelong maintenance of dentition is dependent on saliva's ability to remineralise tooth surfaces which have been subjected to demineralisation from microbially generated organic acids. The remineralisation of tooth surfaces relies on the calcium and phosphate ions in saliva and it has been suggested that overall low levels of these ions in saliva can provide a moderate risk of caries onset (Leone and Oppenheim, 2001). A reduction in the rate of salivary flow is a primary risk factor in oral infections. A reduction in salivary secretion is usually due to the intake of oral medication (Pajukoski *et al.*, 2001), chronic diseases (e.g. Sjögren's syndrome [Leung *et al.*, 2004]) or the sequelae of irradiation for cancer of the head and neck (Dawes and Odum, 2004). All of these circumstances have been shown to increase the risk of oral infections, tooth mineral loss and caries lesion depth (Bardow *et al.*, 2003). The decrease of salivary flow lowers the bicarbonate/carbonate buffering capacity which permits a more rapid decrease in salivary pH due to the presence of bacteria acids and increases the risk of dental caries formation (Diaz-Arnold and Marek, 2002). However, evidence indicates that the salivary buffering capacity alone is not as strong a caries indicator as the overall decreased salivary flow rate (Leone and Oppenheim, 2001). For example, children suffering from familial dysautonomia, where one of the symptoms is an increased flow rate of saliva from salivary glands, exhibit an extremely low level of dental caries, suggesting that the caries free state of these children is most likely due to the high salivary flow rate (Mass *et al.*, 2002).

Gingival crevicular fluid (GCF), much like saliva, contains a complex mixture of substances derived from serum as it passes into the oral cavity at the gingival/enamel margin (see Fig. 1.9). GCF also contains leukocytes, structural cells of the periodontium (the tissue that surrounds, protects and supports the roots of the teeth) and oral microflora. GCF is a clinically significant fluid as it can be used as an indicator for the health or disease of the periodontium (Uitto, 2003).

The involvement of GCF with the dental caries process has received little attention. However, evidence of an adaptive host defence response towards cariogenic bacteria has been detected in the GCF (Smith, 2002).

1.4.2.2 Host diet

The diet of the host has a significant role in the initiation and prolongation of dental caries. More specifically, refined carbohydrates, such as sucrose, have been intensely investigated for their cariogenicity and it is now well understood that they have two major roles to play in dental plaque formation on teeth. First, carbohydrates, particularly sucrose, are involved in adhesion and dental biofilm formation, and secondly, all refined carbohydrates serve as a readily available source of energy for many early colonising bacteria (Brown, 1975; Zero, 2004). Oral bacteria are capable of producing extracellular polymers from dietary sucrose aiding adhesion and biofilm formation (Fig 1.8). This is one reason why sucrose is more cariogenic than its component sugars, fructose and glucose (Mandel, 1979; Rolla, 1989). The mechanisms by which secreted proteins are able to convert sucrose into complex carbohydrate polymers without the input of energy, or to interact with these polymers, have been extensively investigated in streptococcal species, especially in *S. mutans*. These proteins include the glucosyltransferases (Gtfs) and fructosyltransferase (Ftf) enzymes that convert sucrose into glucans and fructans, respectively, as well as the glucan binding proteins (GBPs) (Fig 1.8) (Jacques and Wittenberger, 1981; Aoki *et al.*, 1986; Walker and Schuerch, 1986; Hanada and Kuramitsu, 1988; Ueda *et al.*, 1988; Hanada and Kuramitsu, 1989; Honda *et al.*, 1990; Wexler *et al.*, 1993; Shimamura *et al.*, 1994; Venkitaraman *et al.*, 1995; Tsumori and Kuramitsu, 1997; Fujiwara *et al.*, 1998; Rathsam C, 1998; Monchois *et al.*, 1999).

1.4.2.3 Dental biofilm

The presence of a bacterial biofilm is an important factor in initiating bacterial infection of host tissue in a number of diseases. Fundamentally, biofilms are matrices that can consist of a single microbial species or a mixed consortia of micro-organisms that are reversibly or irreversibly attached to a biological or non-biological surface (Donlan, 2002; Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004).

The availability of powerful microscopic and molecular techniques in conjunction with *in vitro* biofilm and *in vivo* animal models is leading to a better understanding of the intricate architecture, genetic regulation and intra- as well as inter-cellular communication in microbial biofilms (Donlan and Costerton, 2002; Allison, 2003; Marsh, 2004). Studies of dental plaque biofilms are no exception. Dental plaque resides primarily on non-shedding tooth surfaces which, under favourable conditions, primarily act to protect teeth against the colonisation of exogenous pathogenic bacteria. However, as noted, it can directly promote the formation of a carious lesion (Sbordone and Bortolaia, 2003).

Dental biofilms are not unlike the theoretical biofilm model proposed by Donlan and Costerton (Donlan and Costerton, 2002). They are essentially composed of a poly-microbial community consisting of genetically distinct taxa encapsulated in an extracellular matrix composed of nutrients as well as host and microbial factors (Kolenbrander, 2000; Marsh, 2004). The biofilm structure is composed of channels and voids that allow mass transfer of dissolved and particulate substances into and out of the biofilm matrix via the surrounding fluid. In the case of dental plaque on teeth, this fluid is saliva or the GCF (section 1.4.2.1) (Fig 1.9) (Hall-Stoodley *et al.*, 2004). Imbalance of the influx and efflux of nutrients into dental plaque creates a non-uniform gradient that will either be beneficial or detrimental to the host. For example, an increased concentration of fermentable carbohydrate from food begins a cascade of events that leads to a notable lowering in pH as acidic fermentation products accumulate at the dental biofilm/tooth interface. If a low pH is maintained, biofilm homeostasis is disrupted and the population dynamics shifts towards supporting aciduric as well as acidogenic bacteria. These cariogenic bacteria will proliferate and demineralisation of the tooth enamel will be favoured. This cycle is known as the ecological plaque/caries hypothesis that was first postulated by Marsh in 1994 (Marsh, 1994; Bradshaw and Marsh, 1998; Marsh, 2000; Marsh, 2003). Investigations that are centred on deciphering how biofilms shift from health to disease are aimed at developing effective preventative treatments.

1.4.2.4 Microbiological aspect of dental caries

The majority of humans acquire cariogenic bacteria by vertical transmission, predominantly from mother to infant, although some studies have indicated that horizontal transmission between children and other family members can occur (van Loveren *et al.*, 2000; Mattos-Graner *et al.*, 2001; Berkowitz, 2003; Florio *et al.*, 2004). However, the timing of acquisition of cariogenic bacteria such as mutans streptococci, still remains controversial (Berkowitz, 2003; Florio *et al.*, 2004). Nevertheless, the colonisation of potential cariogenic bacteria in humans may be either persistent or transient and can begin from as young an age as 15-20 months (Florio *et al.*, 2004; Klein *et al.*, 2004).

No single bacterial species has been found to be the sole microbial agent responsible for initiating dental caries. As such, dental caries is effectively a polymicrobial infection. It has been estimated that approximately 700 bacterial species, half of which may be uncultivable, are associated with the oral cavity, though not all are associated with dental plaque or a carious lesion (Wilson, 2001; Mitchell, 2003). However, analysis of cultures of human dental plaque associated with enamel carious lesions have identified mutans streptococci, particularly *S. mutans* and *Streptococcus sobrinus*, and lactobacilli as the most frequently isolated bacteria (Hamada and Slade, 1980; Loesche, 1986; van Houte, 1994). These bacteria are important in caries initiation, due to their highly acidogenic properties (van Houte *et al.*, 1996). *S. mutans* has been found to be associated with many forms of carious lesion though it is predominantly isolated from incipient or shallow lesions even though it can be found in some deep carious lesions. In contrast, lactobacilli are chiefly recovered from deep carious lesions (Boyar and Bowden, 1985; Milnes and Bowden, 1985; Nyvad and Kilian, 1990; Hahn *et al.*, 1991; Byun *et al.*, 2004). On the other hand, pleomorphic Gram-positive bacteria, particularly, *Actinomyces*, are considered to play a role in dentinal and root surface caries (Hahn *et al.*, 1991; Brailsford *et al.*, 1999; Bjorndal and Larsen, 2000; Brailsford *et al.*, 2001).

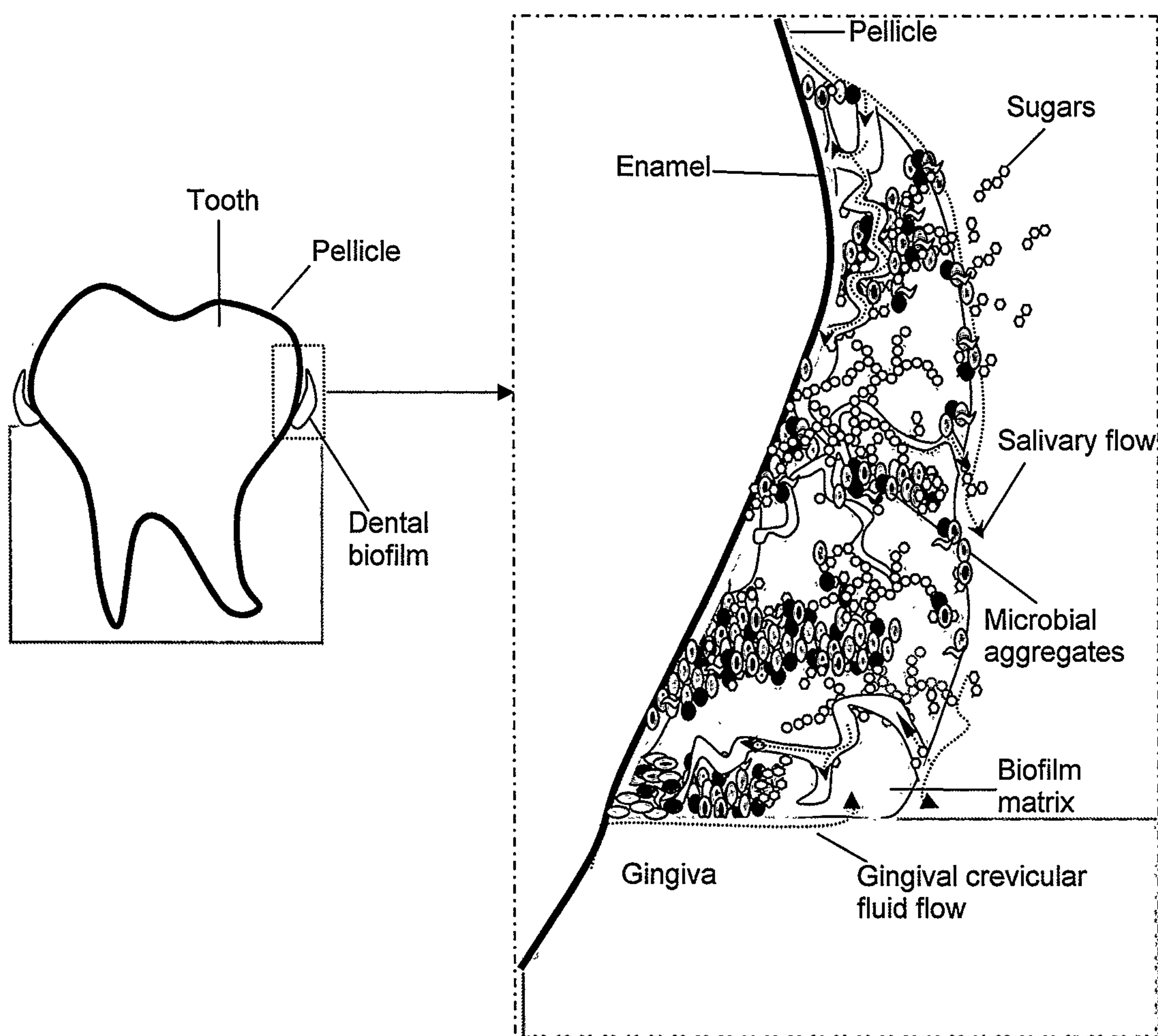


Figure 1.9. General constituents of a dental biofilm on the tooth surface around the gingival margin.

The recent employment of highly powerful molecular techniques, such as 16S rRNA sequencing, has provided an increased awareness of the complex communities of bacterial species associated with dental caries. These techniques have enabled the expansion of the identified group of taxa including novel and presumed uncultivable bacteria associated with the disease (Becker *et al.*, 2002; Munson *et al.*, 2004; Byun *et al.*, 2004; Nadkarni *et al.*, 2004; Chhour *et al.*, 2005). To obtain a comprehensive list of oral microflora associated with dental caries Munson *et al.* (2004) suggested that both traditional culture and molecular techniques must be used concurrently. In this study of Munson *et al.* (2004) the predominant taxa, determined by a molecular approach, were (in descending order of predominance), novel *Propionibacterium* spp., *Olsenella profusa*,

and *Lactobacillus rhamnosus*, while using culture techniques they were, *S. mutans*, *Lactobacillus gasseri/johnsonii* and *lactobacillus rhamnosus* (in descending order of prevalence). More recently, real-time polymerase chain reaction (PCR) analyses specific for *Prevotella* and *Prevotella*-like bacteria have identified the predominance of novel uncultivable bacteria belonging to both taxa in advanced carious lesions (Nadkarni *et al.*, 2004). The employment of molecular tools also increases the stringency and sensitivity of quantitative analysis of oral microflora associated with dental caries (Wall-Manning *et al.*, 2002; Byun *et al.*, 2004). With the combination of powerful molecular tools, traditional culture methods and sensitive quantification methods, the formation of a dental caries ecology model may soon be devised for different stages of the disease. The initiation of the disease, however, still appears to be associated with mutans streptococci, lactobacilli and/or *Actinomyces* depending on the nature of the carious lesion, as noted above.

1.4.3 The role of *S. mutans* in dental caries

Several microbiological studies of human dental plaque led to an association between carious teeth and high numbers of *S. mutans* (De Stoppelaar *et al.*, 1969; Littleton *et al.*, 1970; Loesche *et al.*, 1973). Furthermore, cross-sectional epidemiological studies on randomly sampled human populations also found a positive correlation between the isolation of *S. mutans* from dental plaque and the incidence of dental caries (Loesche *et al.*, 1975; Hamada and Slade, 1980; Loesche, 1986). Recent genotypic analysis of oral biofilms associated with active dental caries suggest that *S. mutans* and other mutans streptococci dominate the microbial population in the habitat adjacent to the caries-active site (Paddick *et al.*, 2003). It is now widely recognised that *S. mutans* is a key aetiological agent involved in initiating the formation of dental caries, and it is the acceptance of this association which has lead to intensive research into the role of this bacterium in the disease.

S. mutans is a Gram positive coccus, although it can appear as short rods or cocco-bacilli, that grows optimally under anaerobic conditions. *S. mutans* is also part of the commensal microflora in both the oral cavity and the upper respiratory tract and is frequently isolated from human

faeces. It is also an aetiological agent in community acquired infective endocarditis (Hardie and Marsh, 1978; Hamada and Slade, 1980; Mylonakis and Calderwood, 2001). *S. mutans* does not play a role in the initial stages of tooth colonisation, which is dependent on pioneering streptococci such as *Streptococcus sanguis*, *Streptococcus oralis* and *Streptococcus mitis*. These streptococci attach to the acquired enamel pellicle (section 1.4.2.1) (Hamada and Slade, 1980; Smith *et al.*, 1993; Pearce *et al.*, 1995). *S. mutans* is therefore considered a secondary coloniser and exists as part of the polymicrobial community of healthy dental plaque which is able to prevent the colonisation of the host dentition by exogenous and potentially pathogenic species (Marsh, 2000). Like all bacteria in dental plaque, *S. mutans* requires endogenous (host acquired) and exogenous (dietary) nutrients to proliferate. Under homeostatic conditions in a healthy individual the numbers of *S. mutans* remain low due to antagonism from other oral bacteria through autogenic succession (community development influenced by microbial factors), which prevent its proliferation (Marsh, 2000). The introduction of a diet rich in carbohydrate, particularly sucrose (see section 1.4.2.2), can shift the dynamics of the microbial community as the early colonising bacteria are unable to metabolise and proliferate as the pH becomes acidic.

Since *S. mutans* is resilient to a low environmental pH and has a strong association with the dental caries process, the virulence mechanisms that allow *S. mutans* to proliferate at low pH and tolerate this environment have been the subject of considerable research. These factors are outlined in the following section.

1.4.4 Cariogenicity factors of *S. mutans*

For *S. mutans* to initiate dental caries it must be able to efficiently adhere and colonise the tooth surface as part of a dental biofilm (section 1.4.2.3) (Brown, 1975; Janda and Kuramitsu, 1976; Burne *et al.*, 1997). *S. mutans* has been shown to possess several important traits that facilitate its formation and association with dental biofilms in the presence of sucrose (Fig 1.8)(Kuramitsu, 1975; Kuramitsu and Ingersoll, 1978; Rolla *et al.*, 1985; Rolla, 1989). Other traits include the ability to adhere to the salivary pellicle (Bleiweis *et al.*, 1990; Bowen *et al.*, 1991) as well as co-aggregate (Tsumori and Kuramitsu, 1997; Vacca-Smith and Bowen, 1998; Banas *et al.*, 2001;

Banas and Vickerman, 2003) with other bacteria already established in dental plaque on the tooth surface. In order to alter the homeostasis of the biofilm to a carious state, or to survive in a consistently low pH environment that favours its proliferation over other Streptococci, *S. mutans* needs to continue to metabolise under low pH conditions and to tolerate the acid produced. These mechanisms are outlined below.

1.4.4.1 Tolerating and adapting to a low pH environment – aciduricity

The capacity of *S. mutans* to prolong growth or increase its acidurance is an important cariogenic trait and is dependent on making significant changes to its cellular physiology (Hamilton and Buckley, 1991; Quivey *et al.*, 2001). To this end it has been shown that *S. mutans* is capable of an adaptive acid tolerance response (ATR) when rapidly exposed to low pH (Belli and Marquis, 1991). The ATR is a complex set of mechanisms by which the bacterium is able to minimise the detrimental affects of a low pH environment and allow it to survive (Ma *et al.*, 1997).

(i) *S. mutans* protein expression under low pH

The ATR of *S. mutans* involves the synthesis of new proteins that enable acid tolerance (Ma *et al.*, 1997; Svensäter *et al.*, 1997). For example, batch culture studies which rapidly exposed several strains of *S. mutans* grown to exponential-phase to pHs as low as pH 4.0 demonstrated that it takes 90-120 min for a fully functional ATR to develop (Svensäter *et al.*, 1997). In comparison with other bacterial species associated with dental plaque, SDS-PAGE analysis showed that *S. mutans* produced the most complex acid-shock protein profile with 36 proteins up-regulated, 25 of which appeared in the first 30 min (Hamilton and Svensäter, 1998). Acid-induced protein expression was further characterised using 2-DGE. It was found that an acid shock from pH 7.5 to 5.5 enhanced the expression of 65 proteins and decreased 24. Furthermore, it was also found that cells stressed by salt, heat, oxidation and starvation prior to acid-shock exhibited a better ATR (Svensäter *et al.*, 2000).

Recently, the proteomes of *S. mutans* cultivated in batch culture from a starting pH 7.0 or pH 5.2, respectively, were compared. Eighteen proteins were found to be up-regulated and 12 down-

regulated during acid tolerant growth. The majority of proteins identified as being up-regulated belonged to the glycolytic pathway, with other proteins being involved in cellular maintenance. Of those that were down-regulated, the majority were stress-induced proteins as well as transport proteins involved in carbohydrate uptake (Wilkins *et al.*, 2002).

(ii) The maintenance and protection response of *S. mutans* to a low pH environment

A number of mechanisms involved in the maintenance and protection of *S. mutans* intracellular components have been investigated. For instance, when the environmental pH becomes acidic, the intracellular pH of *S. mutans* is lowered to less than a favourable pH for the maintenance of DNA integrity, since an acidic cytoplasm can result in the loss of purines and pyrimidines leading to the formation of structurally and functionally unstable forms of DNA (Lindahl and Nyberg, 1972). *S. mutans* appears to possess two mechanisms that respond to repair DNA damage under low acidic conditions, one of which is the Recombinase A, RecA, response which is responsible for catalysing homologous recombination of DNA (Quivey *et al.*, 1995). Evidence for the second mechanism of DNA repair came from the observation that no phenotypic difference was observed between a *recA* mutant and wildtype cells grown at pH 7.0 and pH 5.0, (Quivey *et al.*, 1995). This second DNA repair mechanism showed functional similarity to other DNA repair enzymes and was later identified to possess a 3' to 5' exonuclease activity (Hahn *et al.*, 1999). Differential display PCR identified a gene with increased expression when *S. mutans* was grown at pH 5.0. This gene was homologous to the *uvrA*-UV repair exonuclease gene of *Bacillus subtilis*. A knockout mutant of this gene in *S. mutans* showed greater DNA damage compared with wildtype cells when exposed to low pH, suggesting that *uvrA* and the nucleotide excision repair pathway were involved in adaptation to low pH and were capable of repairing acid damaged DNA (Hanna *et al.*, 2001).

Heat shock proteins of the Hsp70/DnaK group belong to a molecular chaperone system that facilitates the refolding of denatured/unfolded proteins during the imposition of an environmental stress (Lindquist and Craig, 1988). In a preliminary study on the role of DnaK in *S. mutans*, it was found that DnaK was induced under thermal stress (Jayaraman and Burne, 1995). Further studies conducted using various environmental stresses showed that both *dnaK* mRNA and DnaK protein levels in cells grown at a steady state of pH 7.0 or pH 5.0 increased during the ATR and

that these elevated levels persisted in acid adapted cells (Jayaraman *et al.*, 1997). Another molecular chaperone, GroEL, was later found to have a similar increased *groEL* mRNA response by growing *S. mutans* at pH 5.0, but the level of GroEL itself was no different to that at pH 7.0 (Lemos *et al.*, 2001). However, both studies suggest that a significant increase in the level of DnaK synthesis is required at pH 5.0 for the correct biosynthesis of the F₁F₀-ATPase complex, either to enable proper folding or to enable optimal placement of the F₁F₀-ATPase in the membrane (Jayaraman *et al.*, 1997; Lemos *et al.*, 2001). Under acidic growth conditions, the membrane embedded F₁F₀-ATPase of *S. mutans* is involved in the export of H⁺ from the cell in order to minimize cellular damage due to acidification of the cytoplasm (Quivey *et al.*, 2000). The F₁F₀-ATPase maintains the cytoplasmic pH at approximately 0.5 - 1.4 pH units above the surrounding acidic environmental pH, enabling *S. mutans* to better tolerate the destructive effects of a low pH (Bender *et al.*, 1986; Dashper and Reynolds, 1992). Studies have shown that the amount of F₁F₀-ATPase increases approximately 2- to 4-fold as a result of acid tolerant growth (Belli and Marquis, 1991; Hamilton and Buckley, 1991). These results correlate with the transcriptional upregulation of the operon encoding F₁F₀-ATPase in *S. mutans* at low pH (Kuhnert *et al.*, 2004). Furthermore, F₁F₀-ATPase has a relatively low pH optimum of 6.0 (Bender *et al.*, 1986; Sturr and Marquis, 1992), which contributes to the enhanced activity of the enzyme when the cytoplasm becomes acidic, making *S. mutans* more resilient to acid than many other microbial species in dental plaque.

S. mutans is also able to minimise proton permeability especially at pH 5.0 (Bender *et al.*, 1986). This is achieved by a shift in membrane fatty acid ratios to favour long chain mono-unsaturated fatty acids in cells grown under acidic conditions (Quivey *et al.*, 2000; Fozo *et al.*, 2004). Recently it has been found that in *S. pneumoniae* FabM, a *trans*-2, *cis*-3-decenoyl-ACP isomerase, is responsible for the change in fatty acid profile by increasing the proportion of long-chained fatty acids at acidic pH (Marrakchi *et al.*, 2002). A mutant *fabM* strain of *S. mutans* exhibited sensitivity to low pH as well as reduced glycolytic capacity and altered glucose-PTS activity when compared with wildtype cells (Fozo and Quivey, 2004; Fozo and Quivey, 2004). It has been suggested that the change in fatty acid initiated by *S. mutans* at a low pH acts as a protective mechanism, by altering membrane structure (Fozo and Quivey, 2004).

Also associated with membrane structure and function are the highly antigenic lipoteichoic acids (LTA). These are molecules that are bound to membrane glycolipids of most Gram-positive bacteria. A *dltC* mutant of *S. mutans* that produces a defective LTA has been shown to exhibit an acid sensitive phenotype with enhanced proton permeability, suggesting that LTA may also play an important role in acid tolerance (Boyd *et al.*, 2000).

In yet another series of experiments, random transposon mutagenesis of the *S. mutans* chromosome and selection of acid sensitive mutants identified a gene homologous to the *E. coli* diacylglycerol kinase gene (*dgk*) that encodes for a membrane associated lipid kinase (Yamashita *et al.*, 1993). The acid sensitivity of *dgk* mutants indicated the potential importance of the Dgk in acid tolerance as it may be involved in the signal transduction of extracellular environmental signals that would initiate lipid synthesis (Yamashita *et al.*, 1993).

Not all ATR or acid tolerant growth responses are related to modifications of membrane lipids, proton extrusion or DNA repair. For example, a cluster of genes analogous to those that encode for a arginine deaminase system in oral streptococci were identified following the release of the genomic sequence of *S. mutans* (Ajdić *et al.*, 2002). Sequence analysis showed that these genes belonged to an agmatine deaminase system (Burne and Marquis, 2000). Analysis of the operon coding for the agmatine deaminase system showed that ammonia production was increased by two-thirds in cells grown on galactose and agmatine in comparison with those grown on glucose and agmatine (Burne and Marquis, 2000). The optimal pH for ammonia production from agmatine was found to be pH 4.0. This system has been hypothesised to confer acid tolerance to *S. mutans* via the production of ammonia from agmatine (Burne and Marquis, 2000; Griswold *et al.*, 2004). In a similar manner, amino acids generated from degraded proteins under periods of stress, such as combined carbon starvation and acid challenge, can be metabolised to form basic end-products, which directly contribute to the increase in intracellular pH (Svensäter *et al.*, 2001a).

Last but not least, is the observation with the *ffh* gene that encodes a homologue of the 54 kDa subunit of the signal recognition particle, Ffh, that is an essential component for a protein translocation pathway (Phillips and Silhavy, 1992; Honda *et al.*, 1993; Walter and Johnson,

1994). Transposon mutagenesis upstream of *ffh* in the intergenic region of *ylxM-ffh*, resulted in an acid sensitive phenotype in *S. mutans*. Northern blot analysis confirmed that immediately upstream of *ylxM* resided an acid inducible promoter (Yamashita *et al.*, 1993; Gutierrez *et al.*, 1999). A mutational study on *ffh* demonstrated that mutants deficient of Ffh are extremely acid sensitive and the complementation of the mutation resulted in the restoration of acid tolerance indicating that Ffh may be involved with the *S. mutans* ATR (Kremer *et al.*, 2001).

(iii) ATR in biofilm grown cells

Although a number of past investigations into the *S. mutans* ATR have concentrated on the free-living planktonic state in liquid culture, more recently the focus has been directed towards understanding this response in biofilms.

In one series of experiments Li *et al.* (2001) demonstrated that acid tolerance was modulated by cell density. Zhu *et al.* (2001) also demonstrated that an increase in biofilm cell density led to an increase in acid tolerance relative to a low cell density biofilm. Furthermore, the tolerance of intact versus dispersed biofilm cells aged between 1 and 7 days old, demonstrated that there was an increase in acid resistance with an intact biofilm as well as a positive correlation with age consistent with the findings of Li *et al.* (Li *et al.*, 2001; Zhu *et al.*, 2001). McNeill *et al.* (2003 and 2004) not only confirmed the results of these two studies, but also discovered that biofilms were less likely to be affected by acid killing when acid shocked than steady state planktonic culture cells.

As noted above (1.4.2.2; Fig 1.8), streptococcal GTFs polymerize sucrose into extracellular glucans that aid in the adhesion of bacteria in dental plaque (Hudson and Curtiss, 1990). GTFs are therefore considered to be virulence traits of *S. mutans* and have been suggested as potential candidates for the production of vaccines against *S. mutans* (Colby and Russell, 1997; Burne, 1998; Rolla, 1989; Monchois *et al.*, 1999; Michalek *et al.*, 2002; Russell *et al.*, 2004). Such studies are prompted by the finding that the level of GTFs expressed by *S. mutans* is greater in caries-active than in caries-free individuals. In addition, *S. mutans* isolated from caries-active individuals show an increased ability to synthesise insoluble glucans (Perrone *et al.*, 1997). It

has also been shown that a low pH can positively influence the expression of two of the three *gtf* genes of *S. mutans*, *gtfB* and *gtfC*, in a biofilm (Li and Burne, 2001).

Tolerance to low pH also appears to be related to quorum sensing in *S. mutans*. Quorum sensing or “bacterial communication” is a process whereby bacteria secrete signals, known as autoinducers (AI), into the environment and these signals are sensed by the surrounding population of bacteria enabling population control. AIs are sensed by other cells via two-component regulatory systems. A two-component regulatory system consists of a membrane-located receptor or sensor that senses and responds to an external signal. The sensor subsequently interacts with a response regulator within the cell regulating the transcription of target genes (Sturme *et al.*, 2002). Growth of the population is controlled when the concentration of AI expressed extracellularly reaches a crucial threshold and the bacterial community responds collectively to alter gene expression (Henke and Bassler, 2004). Quorum sensing is particularly important for bacteria living in a biofilm (Davies *et al.*, 1998; De Kievit *et al.*, 2001). In Gram-negative and Gram-positive bacteria it is believed that LuxS is responsible for synthesizing a universally recognized cell signal referred to as AI-2 (Surette *et al.*, 1999). LuxS has been identified as possibly possessing a role in biofilm formation (Merritt *et al.*, 2003). Although this role for LuxS in biofilm formation is unclear, it has been demonstrated that the *luxS*-dependent signalling pathway plays a crucial role in regulating tolerance to environmental stresses, such as acid adaptation in *S. mutans* biofilms (1.4.4.2) (Wen and Burne, 2004). Recently, however, under low pH conditions in a biofilm, a fivefold increase in the expression of AI-2 was demonstrated in *relA* mutants of *S. mutans*, which resulted in greater acid resistance. This suggests that *luxS* plays a possible role, not in the formation of a biofilm, but in decreasing acid killing of *S. mutans* within the biofilm (1.4.4.2) (Lemos *et al.*, 2004).

Similarly, it has been suggested that a two-component regulatory system with a histidine kinase (sensor), encoded by *hkl1*, and its associated response regulator, encoded by *rr11*, are involved with co-ordinating biofilm in *S. mutans* (Li *et al.*, 2002). Mutants carrying deletions in either one of the two genes showed a 50-70% reduced biofilm biomass in comparison with the parent strain. Moreover, it was demonstrated that the regulatory system encoded by *hkl1* and *rr11* may also be responsible for acid resistance (Li *et al.*, 2002).

1.4.4.2 Sustaining fermentation under low pH – acidogenicity

The classic *in vivo* study performed by Stephan (Stephan, 1940) demonstrated that the ‘resting’ pH (period of no exogenous refined carbohydrates influx) in caries free individuals was pH 7.2 and in caries active individuals the resting pH was 5.5, highlighting a positive relationship between low plaque pH and caries activity. As noted, a continual supply of refined carbohydrates eventually lowers the environmental pH in dental plaque. This shift to an acidic environment imposes a stress on the resident dental plaque bacteria and in some instances can cause irreversible acid toxicity (Assinder *et al.*, 1995; Dashper and Reynolds, 2000). A low pH environment is beneficial for those bacteria that are able to continue to transport carbohydrates and metabolise them while increasing their capacity to maintain a relatively alkaline cytoplasm by sustaining a pH gradient across the plasma membrane (Bowden and Hamilton, 1998). *S. mutans* not only possesses an ability to tolerate and adapt to a low environmental pH but can also continue to ferment carbohydrates under acidic conditions to a greater extent than other streptococci (de Soet *et al.*, 1989; Takahashi *et al.*, 1997). It is the combination of aciduric and acidogenic properties that are important cariogenic determinants. It is these determinants that allow *S. mutans* to dominate many of the other microflora of dental plaque. By continuing to produce acidic end-products at a low environmental pH, dental enamel is demineralised leading to the formation of an incipient carious lesion (de Soet *et al.*, 1989; Minah and Loesche, 1977b; Scheie *et al.*, 1984; Takahashi and Yamada, 1999; van Houte *et al.*, 1991; Harper and Loesche, 1983).

Glycolytic activity in *S. mutans* has been shown to require an intracellular pH optimum of 7.0 and decreases to zero as it approaches pH 5.0. As noted (see 1.4.4 [ii]), *S. mutans* maintains a transmembrane gradient to prevent the cessation of glycolysis at a low external pH of 5.0 by keeping the internal pH approximately 0.5 - 1.4 pH units higher (Bender *et al.*, 1986; Dashper and Reynolds, 1992). The inhibition or damage of the F₁F₀-ATPase activity can decrease or even prevent glycolysis as the cytoplasmic pH drops below 5.0 in *S. mutans* (Iwami and Yamada, 1980). F₁F₀-ATPase is responsible for the maintenance of a higher internal cellular pH and aids in the continuation of glycolysis in addition to the prevention potential toxic effects of H⁺ (Carlsson and Hamilton, 1996; Quivey *et al.*, 2000).

Glycolysis, however, can lead to the production of different acids and these can affect enamel demineralisation to different degrees. The production of different acids is also pH dependent. For instance, when glucose-limited continuous cultures of *S. mutans* are grown at pH 7.0 to mimic conditions in the oral cavity during fasting periods in between meal times, formate and acetate are preferentially produced. In contrast, at pH 5.5 the main product is lactate (Iwami *et al.*, 1992). In addition, when glucose is not limiting in continuous culture, *S. mutans* produces lactate as the major metabolic end-product (Carlsson and Griffith, 1974; Yamada and Carlsson, 1975b). Lactic acid has been demonstrated *in vitro* to confer a higher rate of human tooth enamel demineralisation than other metabolic acid end-products (Margolis *et al.*, 1999).

1.5 Comparative bacterial proteomics

Comparative proteomics is used to examine and compare the differentially expressed proteins between different cellular states of a biological system or between at least two biologically distinct conditions (Cordwell *et al.*, 2001). It also provides information on which proteins are up-or down-regulated or uniquely expressed under a given condition (van Bogelen, 1999). Comparative proteomics has been one of the most prominent tools of choice to investigate and compare bacterial gene expression, especially in pathogenic bacteria (Fraser *et al.*, 2000; Jungblut, 2001). It provides an extensive approach to identifying proteins that are involved with virulence and is also capable of revealing suitable targets for novel vaccines and therapeutics, markers for diagnostics, as well as enabling the identification and differentiation of microorganisms at a sub-species level (Fraser and Rappuoli, 2005). Comparative proteomics can be applied to intra-strain and inter-strain comparisons of bacteria. Intra-strain proteome comparisons are exemplified by growth of the strain under one condition and comparison of its proteome with that derived from another condition. Inter-strain proteome analysis enables unique protein markers to be identified that are particularly useful for comparing strains that can not be distinguished using conventional methods. Comparative proteomics has also enabled discovery-driven investigations into bacterial virulence, with the aim of providing a comprehensive biological understanding of pathogenesis at the protein level.

1.5.1 Proteomics applied to *S. mutans*

A number of studies have used proteomics to evaluate acid tolerance in *S. mutans*. Prior to the completion of the sequencing and annotation of the *S. mutans* genome, the first comparative protein profile of *S. mutans* was published (Hamilton and Svensäter, 1998). This study investigated changes in proteins following environmental acidification using a simple one dimensional SDS-PAGE approach. Cells cultured at pH 7.5 were compared with acid shocked cells at pH 5.5. Thirty-six proteins were found to be acid regulated, but only putative identifications were made based on M_r (Hamilton and Svensäter, 1998). In a second study, the pH stress response was further characterised using 2-DGE. In this instance, an acid shock from pH 7.5 to 5.5 resulted in up-regulation of 64 proteins and the down-regulation of 49 others. Of these proteins, 25 were also found to be uniquely expressed following acid shock. However, no protein identifications were made (Svensäter *et al.*, 2000).

In another study, 2-DGE revealed that fresh *S. mutans* isolates could elicit an ATR under carbon starvation in complex media whereas laboratory strains could not (Svensäter *et al.*, 2001a). Furthermore, by assuming the identification of proteolytic enzymes based simply on M_r s alone, it was suggested that cells supported amino acid/peptide uptake during early stationary phase and produced basic end-products which could increase the intracellular pH (Svensäter *et al.*, 2001a). While it was clearly demonstrated that a significant number of proteins were altered in their level of expression during growth under acidic conditions, putative identification of proteins by comparison of M_r s was insufficient to confidently identify the proteins involved.

The use of insertionally inactivated mutants coupled with 2-DGE have also been used to analyse and characterise the role of specific proteins in the ATR of *S. mutans*. In one study, a repressor protein expressed by *hrcA*, a gene belonging to the partially characterised *dnaK* operon involved with the ATR, was insertionally inactivated (Jayaraman *et al.*, 1997). Comparative 2-DGE analysis of the *hrcA* mutant and the wildtype showed that in the mutant, the molecular chaperone, GroEL, and 10 other unidentified proteins were up-regulated, while the molecular chaperone, DnaK, was down-regulated at least 2-fold during the ATR. The mutant phenotype was sensitive to acid killing and could not lower the pH as efficiently as the wildtype strain,

suggesting that HrcA was partially responsible for regulating one aspect of *S. mutans*' ability to tolerate an acidic milieu (Lemos *et al.*, 2001).

In a fourth study, Hk11 was observed to be involved in acid tolerance, suggesting that some form of quorum sensing system was involved (see above 1.4.4.1). 2-DGE revealed that, of the 17 up-regulated proteins present in the wildtype under low pH, 13 were expressed in the mutant, indicating that the 4 proteins that were not detected in the mutant may be responsible for the lowered ability to tolerate an acidic environment (Li *et al.*, 2002).

In a fifth investigation, the gene encoding the proteinase ClpP, which degrades proteins presented to it by Clp ATPases, was inactivated using a molecular approach. In comparison with the wildtype strain, a 2-fold increase in the molecular chaperones, DnaK and GroESL, as well as at least 25 other differentially expressed, but unidentified proteins, were found to be altered in their level of expression under acidic conditions. An increase in expression of both DnaK and GroESL was believed to be due to the accumulation of denatured proteins caused by acidification of the cytoplasm (Lemos and Burne, 2002). The mutation in *clpP* not only had an effect on acid tolerance but also on biofilm formation (Lemos and Burne, 2002), similar to the situation with a *luxS* mutant (Lemos and Burne, 2002; Wen and Burne, 2004). Such mutational studies in combination with proteomic analyses, not only showed the importance of particular genes in acid tolerance, but also indicated that other proteins were involved.

While the sequencing of the *S. mutans* UA159 genome was in progress, a comparative proteome approach was taken to elucidate the proteome phenotype of *S. mutans* in batch culture at a physiological starting pH of 7.0 and an acidic starting pH of 5.2 (Wilkins *et al.*, 2002). A 2-DGE and MALDI-TOF proteome analysis in conjunction with translated contigs from a commercial *S. mutans* genome sequence not available to the general scientific community were used to identify the protein profiles of the cells grown under each pH regime (see 1.4.4.1). While the initial pHs of the cultures were pH 7.0 and pH 5.2, their terminal pHs were pH 5.3 and pH 4.5, respectively. Under general batch culture conditions environmental variables are not controlled and so can have effects on the protein phenotype independent of the variable under investigation. Proteomic studies using bacteria cells grown in batch culture are therefore not ideal (see 1.3). It was

evident that the cells cultivated at the starting pH 7.0 endured an acid stress response as the general stress-induced protein, DnaK, was up-regulated. This is a common feature under acidic growth conditions (1.4.4.2) (Jayaraman *et al.*, 1997). Thus, unless all variables, with the exception to the variable being investigated, are controlled, a true representation of protein changes due to that variable is not possible.

The proteome studies described above investigated the protein expression patterns of *S. mutans* altered under acidic conditions. Some of these studies focused on particular proteins and their expression patterns, or, alternatively, the effect on overall protein expression in the absence of a particular protein. All of these studies showed that other proteins were simultaneously differentially expressed, even though in most cases none were characterised. This meant that the breadth of the response could not be completely appreciated. Clearly, the identification of the remaining unidentified proteins would have provided a more comprehensive understanding of the changes occurring in the acid-tolerant phenotype, enabling a far more comprehensive characterisation of the response.

1.6 Scope of the research presented in this thesis

It is clear from epidemiological research that *S. mutans* is one of the primary aetiological agents involved in the initiation of dental caries. Recognition of its role in dental caries has meant that *S. mutans* is one of the most widely investigated oral pathogens. A considerable amount of research over the past four decades using physiological and reductionist molecular genetic approaches has been directed towards understanding the mechanisms used by this bacterium to colonise and persist in the oral cavity (Kuramitsu, 2003). Among these have been studies into acid tolerant growth, one of the main virulence determinants of the bacterium that allows it to persist in an environment that can actively lead to enamel dissolution. Despite considerable knowledge being gleaned on the nature of the acidogenic and aciduric properties of *S. mutans*, an overall understanding of the acid-induced phenotype remains unknown. The advent of the *S. mutans* genomic sequencing project coupled with available high-throughput proteomics meant that such a comprehensive approach was feasible when the research reported herein was first contemplated in 1999. As a consequence, the primary aim of the research reported in this thesis

was to grow *S. mutans* in an artificial salivary-based medium in a chemostat at pH 7.0 in order to obtain a comprehensive 2-DGE proteome map of the bacterium at steady state. Using this map as a baseline, changes in the phenotype were subsequently determined during acid tolerant growth at pH 5.0 using MS technology to identify proteins separated by 2-DGE. The outcome of these proteome analyses were also used by others in the laboratory as a baseline for understanding the changes in phenotype that occur when *S. mutans* undergoes a planktonic to biofilm transition *in vitro* on saliva-conditioned hydroxyapatite surfaces, thus modelling the growth of *S. mutans* in dental plaque on a tooth surface (Rathsam *et al.*, 2005b;Rathsam *et al.*, 2005c) (Rathsam, 2005, in press).

CHAPTER 2 – General materials and methods

This chapter describes materials and methods that were routinely used during this research. New experimental methods that were developed during the course of this study are outlined in the relevant chapters. All procedures were carried out at room temperature (20-22°C) unless otherwise stated.

2.1 Chemicals, Reagents and Enzymes

All general chemicals, reagents and enzymes used in this research were of analytical grade or of the highest purity available.

Amino acids, vitamins, adenine, guanine, uracil, Brain Heart Infusion (BHI), bacteriological agar, mutanolysin, 2-mercaptoethanol, trichloroacetic acid (TCA), formic acid, acetone, bacterial protease inhibitor cocktail, chloramphenicol, exonuclease III, thiourea, caprylyl sulfobetaine (N-decyl-N, N-dimethyl-3-ammonia-1-propane-sulfonate; SB 3-10) and α -cyano-4-hydroxycinnamic acid: Sigma-Aldrich (Steinheim, Germany).

Defibrinated horse blood: AMYL Media (Sydney, Australia).

PBS (Dulbecco 'A' tablets), pH 7.3: Oxoid (Hampshire, England).

Minisart, syringe filter (filter pore size: 0.20 μ m): Sartorius (Gottingen, Germany)

IPG strips: Amersham Biosciences (Uppsala, Sweden) or Bio-Rad (Hercules, CA, USA).

TBP (97%): Fluka (Buchs, Switzerland).

Urea, CHAPS, carrier ampholytes, Tris, N, N, N', N'-Tetramethylethylenediamine (TEMED), bromophenol blue, CBB G-250: Bio-Rad.

Sypro Ruby fluorescent dye: Molecular Probes (Eugene, OR, USA).

Acetonitrile: Mallinckrodt (Chesterfield, MO, USA).

Modified porcine sequencing grade trypsin: Promega (Madison, WI, USA).

Poros R2 resin: PerSeptive Biosystems (Framingham, MA, USA).

D-Glucose, acetic acid, ethanol, D-Lactic acid / L-Lactic acid and formic acid Enzymatic BioAnalysis / Food Analysis kits: Roche (Mannheim, Germany).

2.2 Bacterial growth conditions and harvesting

2.2.1 Plate culture

Streptococcus mutans LT11 was obtained from the laboratory of Tao *et al.* (Tao *et al.*, 1993) and stored at -80°C frozen in glycerol (25% v/v) broth. When required, a loop full of frozen culture was plated onto BHI and/or Horse Blood Agar (HBA) plates (Appendix 1), and incubated under anaerobic conditions (95% N₂, 5% CO₂) at 37°C for 48 h.

2.2.2 Continuous culture

A single colony of *S. mutans* LT11 was taken from HBA plate and used to inoculate 20 mL of sterilized pre-warmed (37°C) modified Defined Mucin Medium (DMM [Appendix 1]) devoid of mucin taken from the chemostat (Sissons *et al.*, 1991) (Bioflow, New Brunswick Scientific Co., Inc, New Brunswick. N. J, USA). The inoculated 20 mL of DMM was then vortexed and incubated under anaerobic conditions (95% N₂, 5% CO₂) at 37°C for 16 h.

The culture was then used to inoculate a chemostat, with a working volume of 335 mL. The chemostat was stirred at 420 rpm and maintained at a constant temperature of 37°C ± 0.1°C and pH of either pH 7.0 ± 0.1 or pH 5.0 ± 0.1 with the addition of a mixed alkali solution of 1M

KOH and 1M NaOH. The chemostat was maintained under anaerobic conditions (95% N₂, 5% CO₂) at a D of $0.100 \pm 0.001 \text{ h}^{-1}$.

When steady state had been achieved (~ 7 mean generations [1 generation = 7 h]), the contents of the culture vessel were harvested by centrifugation (10 000 x g , 15 min, 4°C) in the presence of 50 μg chloramphenicol mL^{-1} and 2.5 μL bacterial protease inhibitor cocktail mL^{-1} , washed twice in cold PBS (pH 7.8) containing the same inhibitors. The culture supernatant was separated and processed according to the procedures described in section 2.4.2 and the cell pellet lyophilised. All continuous cultures were grown in triplicate.

2.3 Gram Staining

Gram-staining was used to confirm the purity of *S. mutans* cultures (Lillie, 1954). *S. mutans* was smeared onto glass microscope slides and fixed onto the slides by flaming the non-smeared side with a naked blue flame. The fixed smears were then stained with crystal violet solution for 1 min and rinsed well with running tap water on the reverse side until water was running clear. The slides were then completely covered with Gram's iodine solution and left to stand for 2 min and gently decolourised by rinsing with 70% (v/v) ethanol for 30 sec. Slides were counterstained using 0.5% (w/v) basic fuchsin in 18 M Ω H₂O for 1 min. The slides were rinsed with tap water, thoroughly dried and examined under a microscope using oil immersion lens at 100 x power.

2.4 Protein isolation

2.4.1 Preparation of cellular proteins

Lyophilised cells (10 mg) were re-suspended in 1 mL of 10 mM K₂HPO₄ containing 10 mM MgCl₂ and 1 mM CaCl₂ and incubated at 60°C with 500 U mutanolysin mL^{-1} for 40 min. The whole cell lysate was exhaustively dialysed against 18 M Ω H₂O containing 0.05% (w/v) NaN₃, lyophilised, and the proteins extracted and precipitated using a modified TCA-acetone procedure (Granier, 1988). Briefly, 1 mL of protein precipitating agent, consisting of 10% (w/v) TCA and

0.07% 2-mercaptoethanol dissolved in ice cold acetone was used to resuspend the lyophilised cell lysate. The resuspended cell lysate was incubated at -20°C for 1 h. Precipitated proteins were centrifuged (12,000 x g, 80 min, -4°C) and the supernatant discarded. The pellet was rinsed with 1 mL of ice cold acetone supplemented with 0.07% (v/v) 2-mercaptoethanol and vacuum dried in a speed-e-vac (Savant Instruments, Holbrook, NY, USA) for 1 h. The precipitate was resuspended in 1 mL of 2-DGE-solubilising solution or 'multiple surfactant solution' (MSS) consisting of 5 M urea, 2 M thiourea, 2 mM tributylphosphine, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 0.2% (v/v) carrier ampholytes (pH 3-10), 40 mM Tris and 0.002 % (w/v) bromophenol blue. The sample was sonicated (Branson, Danbury, USA, output control of 2, 10 x 10 s, 20–22°C with cooling on ice between each burst) before being incubated at room temperature (20–22°C) for 20 min with 150 U of exonuclease III to degrade any DNA. The solubilized proteins were separated from non-solubilized cellular components by centrifugation (12 000 x g, 8 min, 15°C) and either used immediately or stored at -80°C until required.

2.4.2 Preparation of extracellular proteins

The extracellular culture fluid was filtered (MF75 filter units, Nalge Nunc International, Rochester, NY, USA) and the proteins recovered using a modification of the method of Chu *et al* (2000). In essence, culture fluid proteins were precipitated overnight at 20°C with 10% (w/v) TCA before being centrifuged and washed in ice-cold acetone (17 000 x g, 30 min, 4°C). After air-drying, the proteins in the pellet were solubilized as described above.

2.5 Protein separation

2.5.1 First dimension – isoelectric focusing

Cellular proteins extracted from both 0.5 and 0.75 mg dry wt of cells were dissolved in 450 µL of 2-DGE-solubilising solution and either loaded by in-gel rehydration onto pH 4-7 IPG strips or cup-loaded onto narrow range (pH 4.0-5.0, 4.5-5.5 and pH 5.5-6.7) or basic pH 6-11 IPG strips pre-swollen with 350 µL of 2-DGE MSS. For the analysis of the culture fluid, a 13-fold

concentration or approximately 100 µg of precipitated protein from each of the three chemostats was dissolved in 460 µL of 2-DGE MSS and the solution used to rehydrate pH 4-7 IPG strips.

Proteins were separated and focused in the first dimension according to their *pI* using the running conditions indicated in Table 2.1 A, B and C, unless otherwise stated in the relevant chapters. The cellular and culture fluid proteins were focused in triplicate on broad range pH 4-7, and in duplicate on narrow range IPG strips (as appropriate) using a Multiphor II (Amersham Biosciences, Uppsala, Sweden) at 20°C. Post isoelectric focusing strips were either used immediately or stored at -80°C.

Table 2.1 (A) IPG pH 4-7 isoelectric focusing programme

Steps	kV	Time (h)	kVh
1	0.1	3	0.3
2	0.3	2	0.6
3	1	2	2
4	2.5	2	5
5	3.5	18	63
6	0.1	2	0.2
Total		29	71.1

Table 2.1 (B) IPG pH 6-11 isoelectric focusing programme

Steps	kV	Time (h)	kVh
1	0.1	2	0.2
2	0.3	5	1.5
3	1	2	2
4	2.5	2	5
5	5.0	15	75
Total		26	83.7

Table 2.1 (C) IPG narrow range (1 pH unit) isoelectric focusing programme

Steps	kV	Time (h)	kVh
1	0.1	7	0.7
2	0.3	6	1.8
3	1	2	2
4	2.5	2	5
5	3.5	2	7
6	5.0	25	125
Total		26	141.5

2.5.2 Second dimension – SDS-PAGE

Focused IPG strips were treated to an equilibration step which involved solubilising and binding of SDS to the focused proteins as previously described (Nouwens *et al.*, 2000) in preparation for separation of the proteins in the second dimension based on their M_r using SDS-PAGE. Essentially, IPG strips were placed in IPG re-hydration trays and each strip layered with equilibration buffer consisting of 6M urea, 2% (w/v) SDS, 20% (w/v) glycerol, 75 mM Tris-HCl pH 8.8, 2.5% (w/v) acrylamide and 5mM tributylphosphine. All equilibration buffer components were solubilised using 18 MΩ H₂O in a water bath sonicator, with the exception of tributylphosphine. Tributylphosphine was prepared separately as a stock solution of 200 mM in isopropanol and stored under N₂ conditions at 4°C and was the last addition to the solution. IPG strips were equilibrated for 30 min on a rocking platform.

Solubilised and denatured proteins were then separated on large format (190 x 190 x 1.5 mm) 10-18% T gradient SDS-PAGE gels using a Protean II xi system (Bio-Rad) according to Laemmli *et al.* (Laemmli, 1970). The 10 and 18% T solutions were prepared separately using a premade solution of 40% (w/v) acrylamide supplemented with crosslinker, 2.6% (w/v) N,N'-methylene-bis acrylamide (Bio-Rad), 75 mM Tris-HCl pH 8.8, 35% (v/v) glycerol (for 18% solution only). Both acrylamide solutions were placed under vacuum for 30 min to remove any dissolved air and subsequently supplemented with 0.0165% (v/v) TEMED and 0.05% (w/v) ammonium persulphate to accelerate acrylamide polymerisation (16 h). IPG strips were embedded on top of

the gradient gels using 0.5% (w/v) agarose containing 0.001% (w/v) bromophenol blue. Proteins were separated by SDS-PAGE at 5mA/gel for 2 h and 20mA/gel for 16 h at 4°C.

2.6 Protein detection and visualisation

Following SDS-PAGE, proteins were fixed in 10% (v/v) methanol and 7% (v/v) acetic acid by incubating the gels for 1 h. Gels were then stained with Sypro Ruby for 24 h and destained with fixative solution (10% [v/v] methanol and 7% [v/v] acetic acid) for imaging with a Molecular Imager Fx (Bio-Rad). Preparative gels were 'double stained' (Cordwell *et al.*, 2002), with CBB G-250 for 24 - 48 h and destained in 3 changes of 18 MΩ H₂O over a period of 16 h. Gels were either prepared for MS analysis (section 2.8) or sealed in plastic and stored at 4°C.

2.7 Image analysis

The number of protein spots on each 2-DGE gel image and the density/volume, or differential expression (DE) value (arbitrary units), of each Sypro Ruby-stained protein spot was determined using the software package z3 and processed according to manufacturers instructions (Compugen, Tel Aviv, Israel). If required, prior to image analysis and image cropping and image noise reduction was undertaken using the 'clean image' tools of the z3 software. The 'clean image' tool removed image contaminants, such as speckling due to Sypro Ruby staining.

Automatic spot detection enabled the differentiation of protein spots from the background and provided information on each individual protein spot co-ordinates, size and density/volume. Automatic spot detection was performed on all gel images. However, if required, manual editing was performed to further decrease image contaminants and enhance the quality of the image used for analysis. DE analyses of equivalent protein spots from triplicate gels from each of three repeat continuous cultures were undertaken. An increase in the level of expression of a protein spot was based on the change in mean DE value.

2.8 Statistical analysis

Dr K Byth Wilson undertook the statistical analysis for chapters 4 and 5. The data were analysed using the statistical software package SPSS for Windows version 12 (SPSS inc, Chicago, IL, USA). Analysis of variance was performed and F tests used to compare the variability within and between groups. A significance level of 5% ($P < 0.05$) was used throughout.

2.9 Protein identification

2.9.1 Protein spot preparation for MS analysis

Protein spots were excised from the gel, placed into individual wells of a microtitre plate and washed 3 times at 37°C with shaking for 1 h with 120 μL of wash solution containing 25 mM ammonium bicarbonate pH 7.8 and 40% (v/v) acetonitrile. The wash solution was removed and gel pieces were subsequently dehydrated in a speed-e-vac. An 8 μL solution of 10ng μL^{-1} Trypsin was added to the dehydrated gel pieces which were left to rehydrate for 1 h at 4°C. Following rehydration, the gel pieces were incubated with shaking at 37°C for 16 h to maximise in-gel enzymatic digestion of proteins. To maximise the release of peptides, the digested proteins were placed in a sonication bath (Branson, Danbury, CT, USA) for 15 min at 20°C. A 1 μL aliquot of each digest was spotted onto a MALDI target plate (Micromass, Manchester, UK), and 0.5 μL of matrix solution (10 mg α -cyano-4-hydroxycinnamic acid per mL 70% [v/v] acetonitrile) layered on top and allowed to dry. In some instances, micro-scale concentration and de-salting was performed using C₁₈ Zip-TipsTM (Millipore, Bedford, MA, USA) or micro-purification columns employing narrow-diameter GelLoaderTM tips (Eppendorf, Hamburg, Germany) packed with Poros R2 chromatography resin (Kusmann *et al.*, 1997; Gobom *et al.*, 1999). Zip-TipsTM and R2 columns were activated with 20 μL of 100% (v/v) acetonitrile and equilibrated with 20 μL 5% (v/v) formic acid. Peptides were eluted from the columns onto a MALDI target plate with 1 μL of matrix solution.

2.9.2 Mass Spectrometry

2.9.2.1 MALDI-TOF MS

PMM analyses of proteins digested with trypsin were performed on a Micromass (Manchester, UK) TOF-Spec 2E or a PerSeptive Biosystems (Applied Biosystems, Foster City, CA, USA) Voyager DE-STR mass spectrometer with trypsin autolysis peptide masses of 842.5 $[M+H]^+$ and 2211.1 $[M+H]^+$ Da being used as internal standards and/or Glu-fibrinogen 1570.68 $[M+H]^+$ Da as an external calibrant. All mass spectra were obtained in reflectron-delayed extraction mode using an accelerating voltage of 20kV. A minimum of 12 spectra, with at least 30 shots /spectrum, were retrieved.

2.9.2.2 MS/MS

Tandem ESI-MS/MS was performed using a Q-tof hybrid quadrupole/orthogonal-acceleration TOF mass spectrometer (Micromass) as previously described (Cordwell *et al.*, 2002). Tryptic digested peptides were concentrated and desalted using micro-purification columns employing narrow-diameter GelLoaderTM tips packed with porous R2 resin (2.9.1). Peptides were eluted from the column directly into a nanoelectrospray needle in 1-2 μ L of 70% (v/v) acetonitrile containing 0.5% (v/v) formic acid. Sample loaded nanoelectrospray needles were mounted in the source and stable flow was achieved where capillary voltage reached between 900-1200V. An initial m/z scan was performed to detect peptide precursor ions and a peptide precursor ion subsequently selected for fragmentation in the collision cell. Argon was selected for gas induced collision dissociation. Both collision energies (18-30 eV) and argon pressure were set for optimal fragmentation conditions. Data were recorded and processed using MassLynx version 3.5 software (Micromass) with the amino acid sequences being derived from the mass differences between the y - or b -ion 'ladder' series.

2.9.3 Data processing and protein databases

Peak list generation made use of either Data ExplorerTM (PerSeptive Biosystems) or ProteinLynx version 3.4 (Micromass) software. All database searching with PMM made use of the ProteinLynx software package containing the unpublished 6 contigs of the *S. mutans* UA159 genome (<http://www.genome.ou.edu/smutans.html>) downloaded on October 6, 2001 that were translated in all 6 reading frames using the Flip6Frames program (Brossard, 1997; <http://megasun.bch.umontreal.ca/ogmp/ogmpid.html>) accessed through the Australian Genomic Information Service (<http://www.angis.org.au/> WebANGIS). Parameters for protein identification included a mass tolerance of 150 ppm and a maximum of 1 missed cleavage per peptide while taking into consideration methionine sulfoxide and cysteine acrylamide modification. Matches were defined on the basis of the number of matching peptide masses and the total percentage sequence covered by the peptides. As a general rule, at least 3 matching peptides and a minimum total sequence coverage of 25% was taken to match a given translated ORF with confidence, with the proviso that cleavage products and low and high mass proteins did not always conform to this rule. All translated ORFs that matched PMM data were then used to query the annotated *S. mutans* genome at the Oral Pathogen Sequence Databases (<http://www.stdgen.lanl.gov/oragen>; Ajdić *et al.*, 2002), using the local BLAST search facility to determine the gene identification number. The original six contigs were used in this manner as some genes identified in these contigs were not present in the final annotated version (Ajdić *et al.*, 2002).

2.10 Amino acid analysis

The concentrations of free amino acids in spent medium were analysed using pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate using a Waters AccQFluor reagent kit (Waters Corp, Milford, MA, USA ; [Cohen and Michaud, 1993; Cohen and De Antonis, 1994]). Amino acid derivatives were separated and quantified by reversed phase (C₁₈) using a Waters AccQTag column (15 cm x 3.9 mm i.d.) according to the manufacture's protocol (Cohen, 2000). The high performance liquid chromatography (HPLC) consisted of a Waters Alliance 2695 Separation Module, a Waters 474 Fluorescence Detector and

a Waters 2487 Dual 1 Absorbance Detector in series. The control and analysis software was Waters Empower Pro Module. Tryptophan was analysed by UV detection and all other amino acids by fluorescence detection.

2.11 Metabolite analysis

The concentrations of glucose, acetate, ethanol, formate and lactate present in the medium at steady state were measured enzymatically using appropriate detection kits supplied by Roche according to the manufacturer's instructions. The glucose yield (Y_{glc}) was calculated based on the dry weight of cells (g) per mol of glucose consumed. Using Y_{glc} in conjunction with the mol per mol of product formed the ATP yield was calculated based on the assumption of Harty & Handley (1988), where ATP was produced as 2 mol of ATP per mol of acetate formed; 1 mol ATP per mol of lactate; and 1 mol ATP per mol of ethanol formed.

The amount of accumulated intracellular polysaccharide (IPS) was determined by the potassium iodide method of DiPersio *et al.* (1974). Essentially, washed cells grown at pH 7.0 or 5.0 were resuspended in 150 mM KPO_4 buffer pH 7.0 to an OD_{620} of 1.000. Cells were sedimented by centrifugation (10 000 x g, 15 min, 4°C) and resuspended to 20% volume and 100 μL aliquoted collected, supplemented with 30 μL of 5.3 M KOH and boiled for 90 min. Once the aliquots were cooled, 30 μL of 5.3M HCl, 150 μL of 1M KPO_4 buffer pH 7.0, and freshly prepared 0.2% (w/v) iodine in 2% (w/v) KI solution were added. The samples were then mixed thoroughly and a 200 μL aliquot transferred into a microtitre plate. IPS absorbance was determined in a microplate reader (Bio-Rad) at 540 nm and a standard curve was prepared using glycogen.

Chapter 3- *S. mutans* cellular and extracellular proteome profiles expressed during growth under steady state conditions at pH 7.0.²

3.1 Introduction

Several 2-DGE protein analyses of streptococci have been reported (Champomier-Verges *et al.*, 2002). Those involving *S. mutans* have concentrated on physiological aspects associated with its virulence and survival in the oral cavity (see 1.5.1). In a study of the stress response of *S. mutans*, ¹⁴C-labelled proteins from cells grown in batch culture that had been subjected to pH, temperature, salt, oxidative and starvation stresses, were displayed (Svensäter *et al.*, 2000). By examining two-fold changes in protein expression between stressed and control cells, *S. mutans* was shown to respond to adverse environmental conditions by a complex and diverse alteration in protein synthesis. Unfortunately, no proteins were identified in this study (Svensäter *et al.*, 2000). Changes in ¹⁴C-labelled cellular proteins following a transition of *S. mutans* from the planktonic to biofilm state in a biofilm chemostat have also been reported. A total of 694 protein spots were analysed for enhanced or diminished protein expression. Mass spectral analysis resulted in the identification of 41 proteins, some existing as multiple protein spots (Svensäter *et al.*, 2001b). In a third study, changes in protein expression associated with the growth of *S. mutans* at low pH were analysed following comparison of 2-DGE proteome maps of *S. mutans* grown in batch culture at two different pH. Thirty individual cellular proteins differing in expression by more than 1.5-fold were identified, including proteins involved in energy metabolism, cell-division, translation and transport (Wilkins *et al.*, 2002). What was clear from these studies, was that none had resulted in a comprehensive map of the proteins expressed by *S. mutans* (Svensäter *et al.*, 2001b; Wilkins *et al.*, 2002). The publishing of contigs of the *S. mutans* genome on a public database (<http://www.genome.ou.edu/smutans.html>), however, made

² Aspects of the results in this chapter have been published: Len AC, Cordwell SJ, Harty DW, Jacques NA. (2003) Cellular and extracellular proteome analysis of *Streptococcus mutans* grown in a chemostat. *Proteomics* 3(5):627-646.

such an analysis possible and allowed for the identification of proteins displayed on high-resolution proteomic maps derived from cellular and extracellular fractions of *S. mutans*.

This chapter reports the construction of such a proteome map. In order to achieve both a high-resolution and comprehensive proteome map of cellular and extracellular proteins of *S. mutans* at neutral pH a combination of optimal protein isolation/extraction technique and narrow range IPG strips were used.

3.2 Methods

All methods used were as described in Chapter 2 except for the following modifications which arose during ongoing refinement of techniques during the course of this study.

3.2.1 Protein isolation and solubilisation

To find an optimal protein extraction technique for *S. mutans* LT11, 5 methods were trialled. All solubilised protein sample preparations were either used immediately or stored at -80°C until required.

3.2.1.1 Protein extraction using MSS supplemented with 1% (w/v) ASB-14 and 65mM DTT - MAD

Lyophilised *S. mutans* cells (10 mg) were re-suspended in 1 mL of MAD consisting of MSS (2.4.1) supplemented with 1% (w/v) ASB-14 and 65 mM DTT. The sample was then subjected to sonication, and DNA degradation before the solubilised proteins were separated on 2-DGE according to section 2.4.1.

3.2.1.2 Protein extraction using mutanolysin and MAD

Lyophilised *S. mutans* cells (10 mg) were lysed using 500 U mutanolysin mL⁻¹ and the cell lysate subsequently lyophilised (2.4.1).

Protein was isolated and solubilised by treating lysed lyophilised cells with 1 mL of MAD (3.2.1.1).

3.2.1.3 Protein extraction using mutanolysin, TCA-acetone-mercaptoethanol and MAD

Lyophilised *S. mutans* cells (10 mg) were lysed using 500 U mutanolysin and the cell lysate subsequently lyophilised (2.4.1). Lyophilised lysed cells were then subjected to TCA-acetone-mercaptoethanol protein precipitation (2.4.1). Precipitated proteins were then solubilised in MAD (3.2.1.1).

3.2.1.4 Proteins extracted using mutanolysin, tributyl-*n*-phosphate and MSS

Lyophilised *S. mutans* cells (10 mg) were lysed using 500 U mutanolysin and the cell lysate subsequently lyophilised (2.4.1). Proteins were isolated and precipitated from the lyophilised cells using tributyl-*n*-phosphate precipitation (Mastro and Hall, 1999). Briefly, the cell lysate was resuspended in 1ml of tri-*n*-butylphosphate:acetone:methanol (1:12:1) and incubated at 4°C for 90 min. The precipitated proteins were centrifuged (2800 x g, 4°C, 15 min) and the supernatant discarded. The pellet was then washed with 1ml of tri-*n*-butylphosphate:acetone:methanol (1:12:1) and air dried. Precipitated proteins were then solubilised using MSS (2.4.1)

3.2.1.5 Protein extraction using mutanolysin, TCA-acetone-mercaptoethanol and MSS

Lyophilised *S. mutans* cells (10 mg) were lysed, using 500 U mutanolysin, and then subsequently lyophilized (2.4.1). Lyophilised lysed cells were then subjected to TCA-acetone-mercaptoethanol protein precipitation (2.4.1). Precipitated proteins were then solubilised in MSS (2.4.1).

3.2.2 Preparation of extracellular proteins

Extracellular proteins were isolated according to section 2.4.2. For the analysis of the culture fluid, a 13-fold concentration or approximately 100 µg of precipitated protein from each of the three chemostats was dissolved separately in 460 µL of MSS and the solution used to rehydrate pH 4-7 IPG strips.

3.3 Results and discussion

3.3.1 Analysis of the *S. mutans* proteome by 2-DGE

The use of standardised continuous culture conditions and the availability of contigs of the *S. mutans* UA159 genome allowed a cellular and extracellular proteomic map of *S. mutans* LT11 to be constructed. The conditions chosen were those of neutral pH and a bacterial doubling time of approximately 7 h that were consistent with the conditions associated with a resting dental plaque in between meal times (Ellwood, 1976; Jacques *et al.*, 1979). Glucose was chosen as the source of carbon as this carbohydrate catabolite represses the expression of other genes (Simpson and Russell, 1998), thus allowing for future *in vitro* modelling of pathways expressed when the source of carbon is altered, such as when sucrose is ingested during food intake.

A number of different methods for extracting proteins from chemostat grown *S. mutans* were trialled, as oral Gram-positive streptococci are difficult to lyse and require a specific muramidase to disrupt the integrity of the peptidoglycan matrix of the cell wall.

Previously it was demonstrated that the addition of a powerful zwitterionic detergent, ASB-14, to the protein solubilising solution enhances the solubilisation of hydrophobic proteins, such as membrane proteins (Chevallet *et al.*, 1998; Rabilloud *et al.*, 1999; Nouwens *et al.*, 2000). Consequently, the first method of extraction that was investigated used ASB-14 added to MSS and supplemented with DTT (MAD, section 3.2.1.1). Proteins possessing pIs between 5.5-7.0 were well resolved over the range of pH 4.0-7.0 on a 2-DGE display of proteins solubilised with MAD (Fig 3.1). However, proteins within the pH range of 4.0 to 5.0 were not clearly resolved

(Fig 3.1). This display pattern may have been due to non-proteinaceous material interfering with the migration of proteins within the low *pI* range. Furthermore, the lack of resolution could have been due to the incomplete breakdown of the cell wall or other interfering non-proteinaceous materials, such as carbohydrate or lipid, which are known to hinder protein migration (Granier, 1988; Rabilloud, 1996; Mastro and Hall, 1999).

In the second protocol (3.2.1.2), mutanolysin, a specific fungal muramidase, was used to disrupt the integrity of the cell wall (peptidoglycan matrix) (Yokogawa *et al.*, 1975), in order to theoretically expose more proteins. The combination of mutanolysin-treated cells in addition to the solubilisation of proteins using MAD enabled better resolution of proteins over the pH range of 4.0-6.0 (Fig 3.2). However, the majority of proteins within the pH range of 6.0 – 7.0 were poorly resolved when compared with the use of MAD alone (Fig 3.1).

To remove potentially interfering constituents, such as carbohydrate and salts, a TCA-acetone-mercaptoethanol procedure was included in the protocol (3.2.1.3). A further enhancement of protein resolution was noted over the IPG pH range of 4.0 -7.0 (Fig 3.3). However, a prominent detergent front was present in the lower M_r range (< 20 kDa), a pattern which is commonly detected in 2-DGE following Sypro Ruby staining, when a protein extraction solution contains ASB-14 (Dr S Cordwell, personal communication). This detergent front had clear implications for the running pattern of the lower M_r proteins, since it was often noted that a protein migrating to the area of the detergent front was present in a split or warped protein pattern.

In order to overcome this problem, a fourth protocol was investigated based on the observation that Tributyl-*n*-phosphate combined with acetone and methanol facilitated the delipidation of biological samples and the precipitation of proteins (Horowitz *et al.*, 1985; Granier, 1988). Consequently, mutanolysin treated cells were delipidated using this procedure and subsequently solubilised using MSS (3.2.1.4).

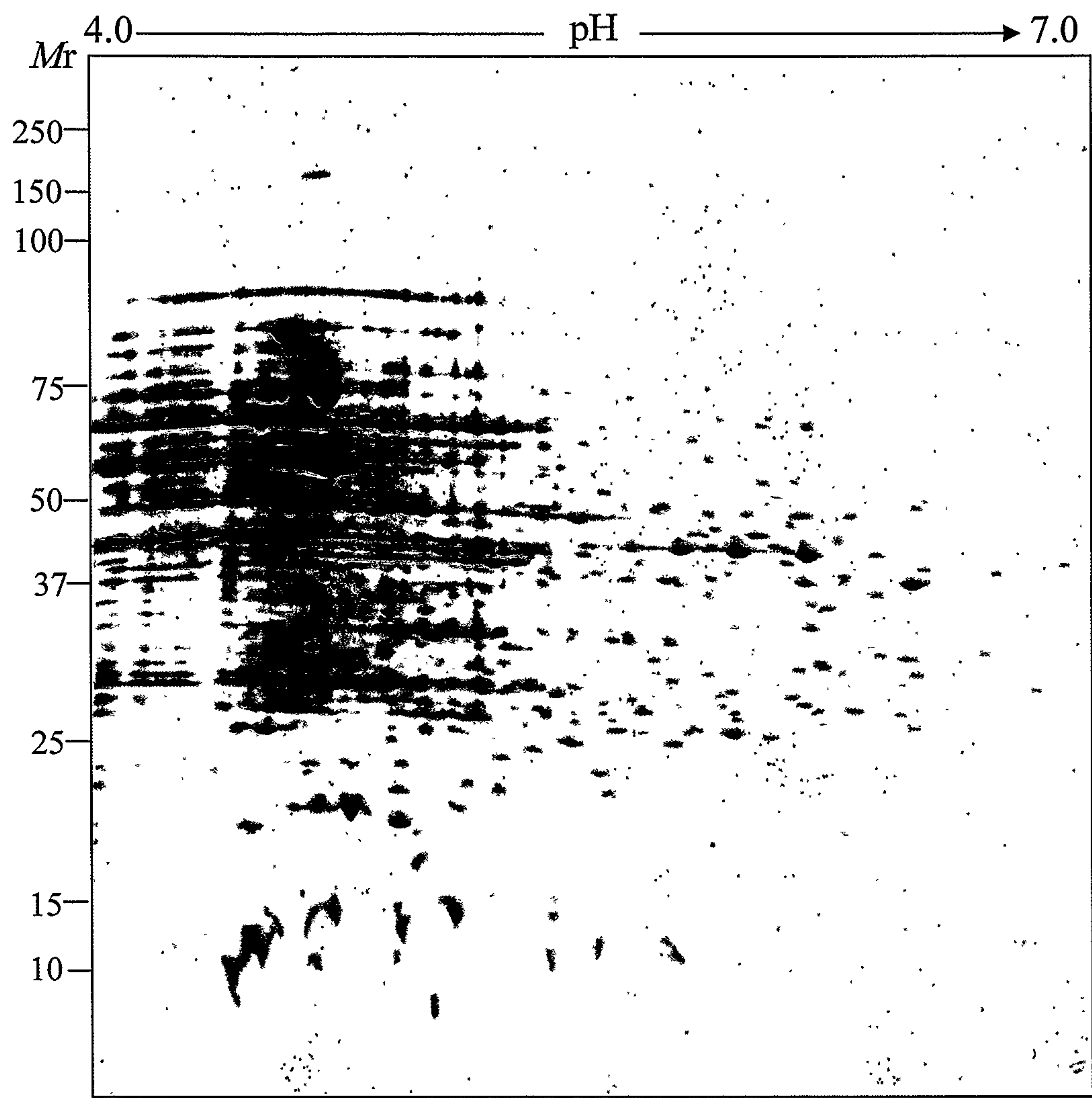


Figure 3.1. 2-DGE proteome map of proteins from *S. mutans* LT11 extracted and isolated with MSS supplemented with ASB-14 and DTT (MAD).

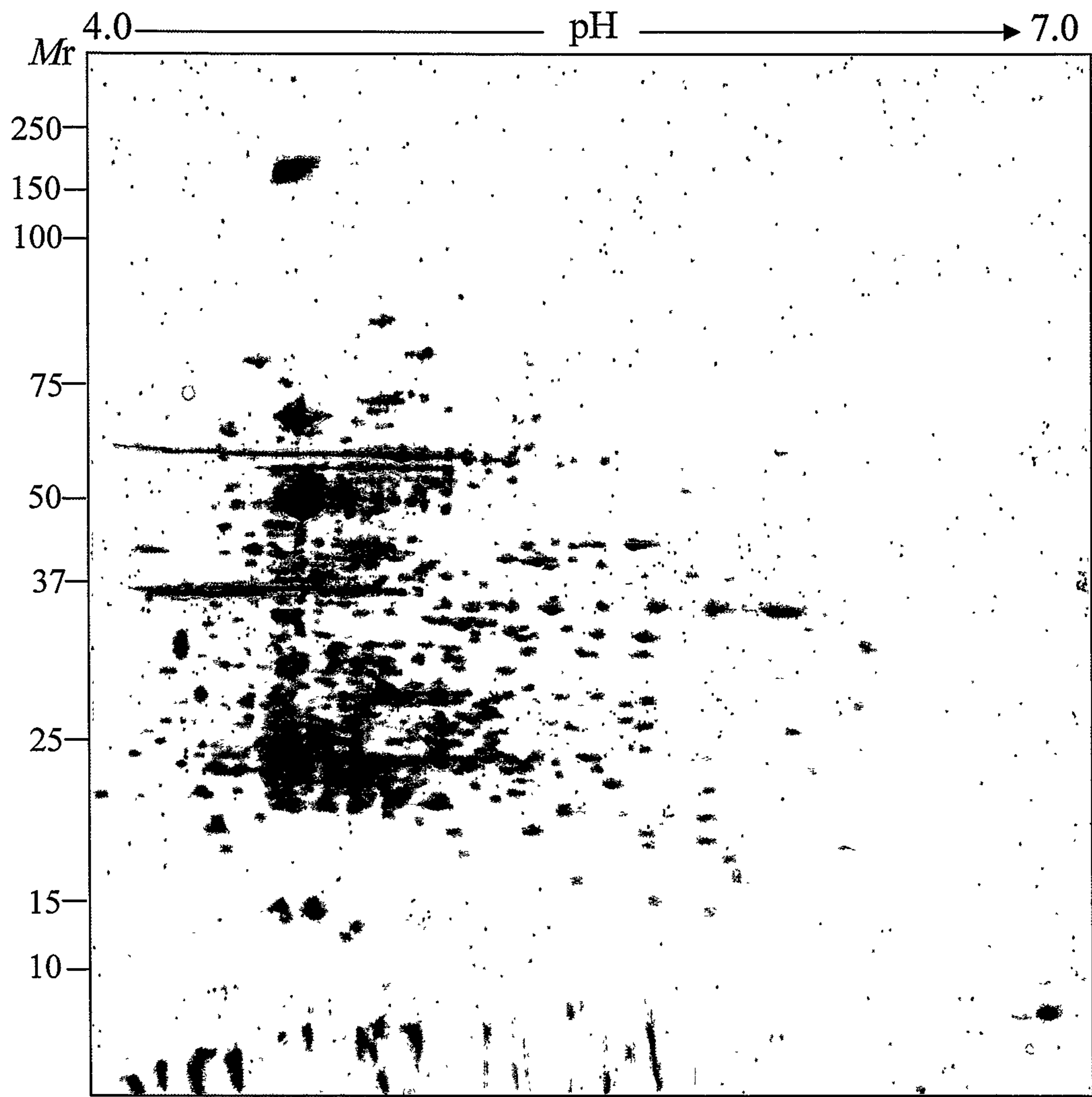


Figure 3.2. 2-DGE proteome map of *S. mutans* LT11 treated with mutanolysin followed by protein extraction with MSS supplemented with ASB-14 and DTT (MAD).

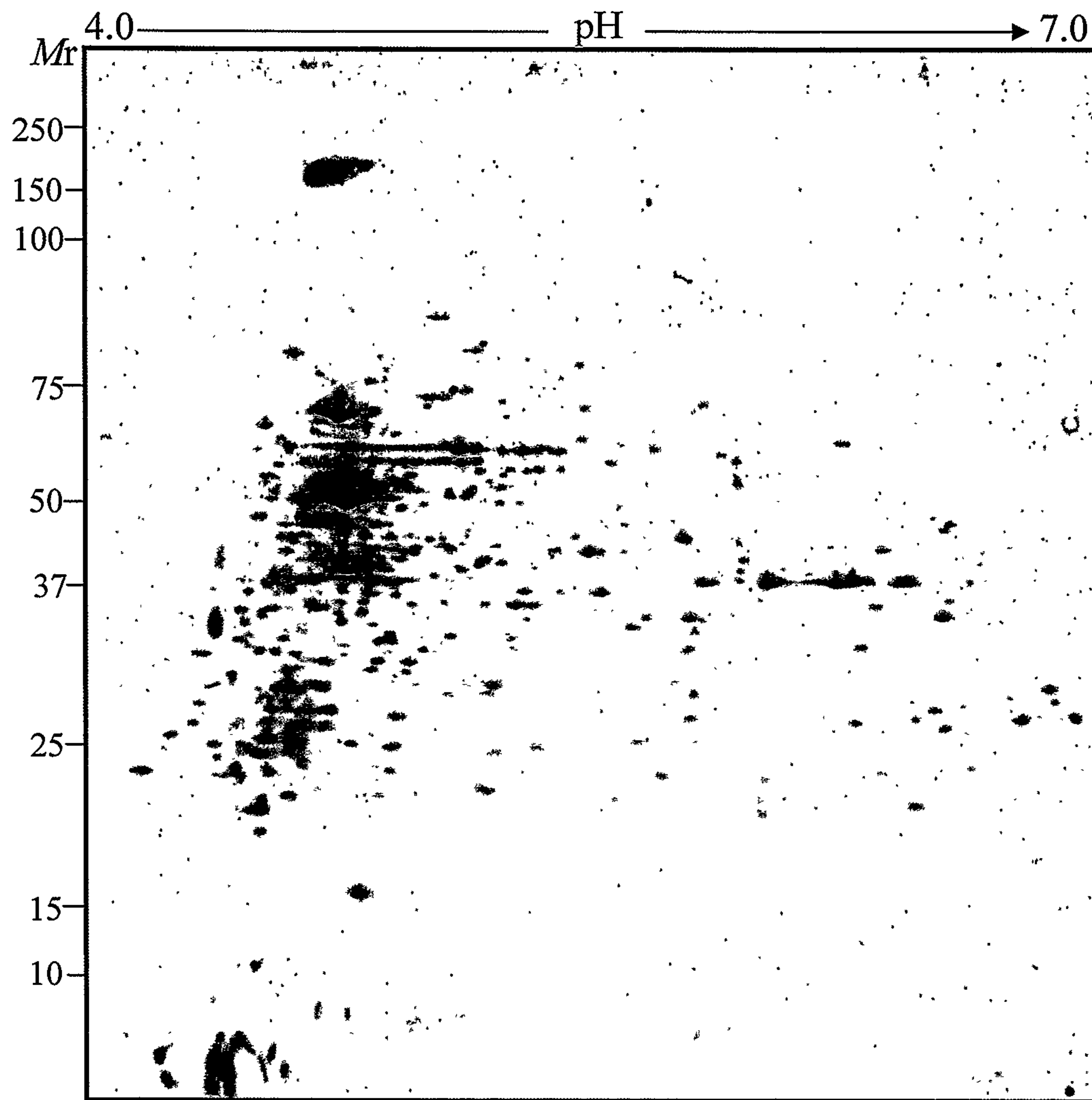


Figure 3.3. 2-DGE proteome map of *S. mutans* LT11 lysed with mutanolysin and the proteins precipitated with TCA-acetone prior to solubling with MSS supplemented with ASB-14 and DTT (MAD).

However, horizontal streaking was prominent within the pH range of 4.0 to 5.5 (Fig 3.4). This result indicated that the major cause of interference in protein separation was not due to contamination of samples with lipids. However, the detergent front was no longer prominent as ASB-14 had been omitted from the protein solubilising solution. The final protocol that was adopted for 2-DGE proteome mapping that provided a high resolution of protein spots made use of mutanolysin, followed by TCA-acetone extraction prior to protein solubilization with MSS (3.2.1.5; Fig 3.5).

3.3.2 Image analysis of 2-DGE proteome map of *S. mutans*

Image analysis, using the software package z3 (2.7), of triplicate 2-DGE gels displaying cellular proteins derived from 3 separate chemostat cultures showed little pattern deviation that could be attributed to either sample or run-to-run variation. The total number of protein spots visible on the 2-DGE gels was approximately 1050 and 200 over the pH ranges 4.0-7.0 and 6.0-11.0 respectively (Fig. 3.6). Protein resolution was further enhanced using narrow pH range (pH 4.0-5.0, 4.5-5.5 and pH 5.5-6.7) IPG strips to resolve clusters of proteins with near identical pI/s in the acidic pH range of 4.5-6.0 (section 2.7). The number of protein spots detected by z3 software on pH range 4.0-5.0, 4.5-5.5 and 5.5-6.7 2-DGE gels was of the order of 800, 1300 and 650 respectively, or approximately twice the number of protein spots detected in the corresponding regions of the pH 4-7 2-DGE gels after allowing for overlapping regions (Fig. 3.6). A similar observation was made following 2-DGE analysis of *Staphylococcus aureus* where the use of narrow range IPG strips increased the number of protein spots that were resolved by 45% (Cordwell *et al.*, 2002). Soluble proteins present in the culture fluid were also focused over the pH range 4.0-7.0. Image analysis revealed highly reproducible 2-DGE gels displaying approximately 900 protein spots (Fig. 3.6).

3.3.3 Identification of *S. mutans* proteins

A total of 502 protein spots that could be visualised by eye following double staining in Sypro Ruby and CBB G-250 were obtained from the cellular and extracellular 2-DGE gels over the pH

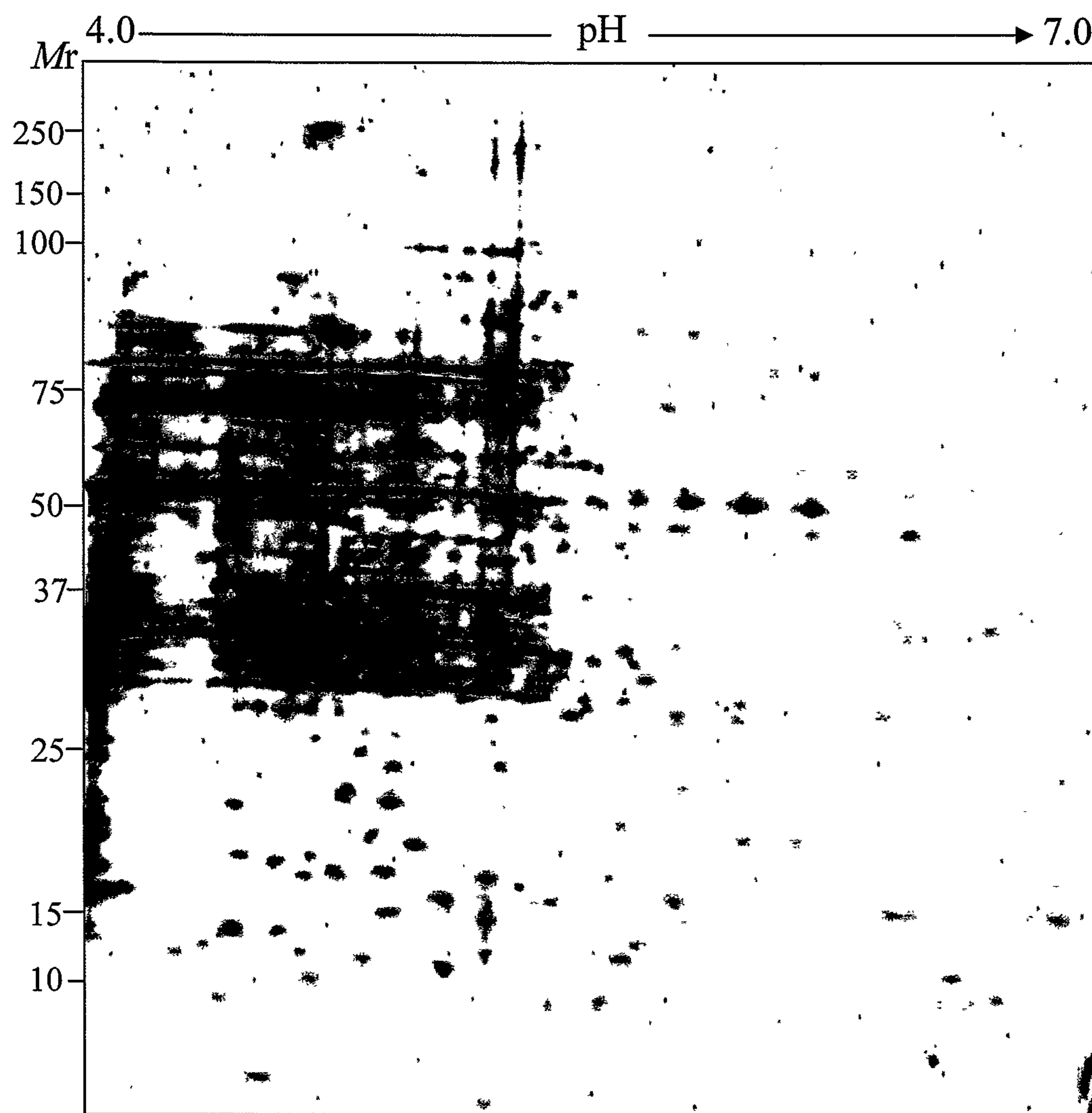


Figure 3.4. 2-DGE proteome map of *S. mutans* LT11 lysed with mutanolysin and the proteins precipitated with tributyl-*n*-phosphate prior to solubiling with MSS.

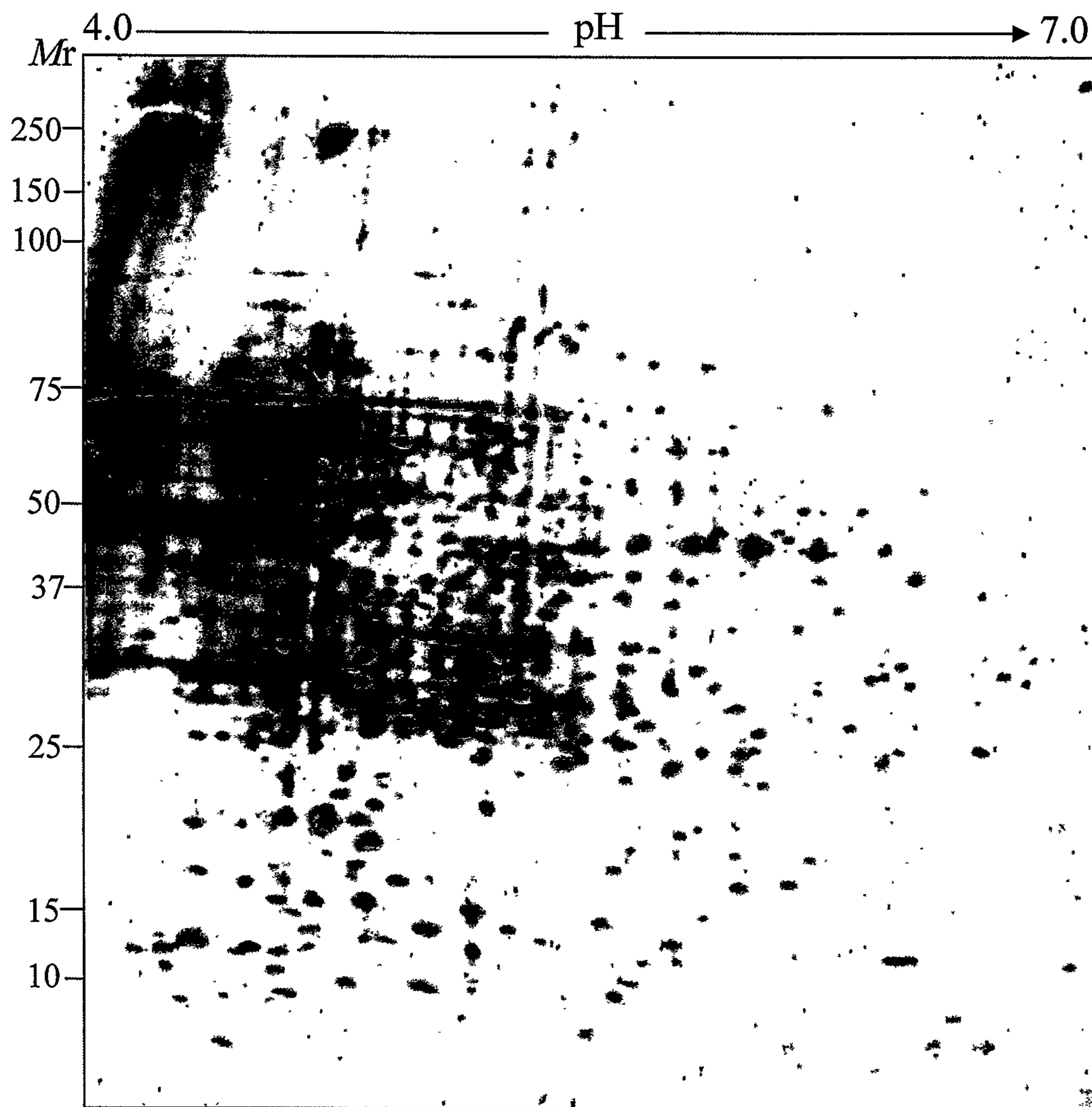


Figure 3.5. 2-DGE proteome map of *S. mutans* LT11 lysed with mutanolysin and the proteins precipitated with TCA-acetone and subsequently solubilised with MSS.

ranges 4.0-11.0 and 4.0-7.0 respectively (Figs. 3.7 and 3.8). All 502 proteins were digested with trypsin and analysed by MALDI-TOF MS (section 2.9). Of these proteins, 416 (313 cellular and 103 in the culture fluid) or 83% provided PMM spectra of sufficient quality to perform a database search of the translated ORFs of the *S. mutans* contigs. The protein spots that could not be characterised were either of insufficient concentration for analysis following purification, or the number of peptides generated by trypsin and characterised by MALDI-TOF MS were insufficient to positively identify the protein. The majority of these proteins were of low abundance and low M_r .

The 416 proteins that were identified by PMM matched 197 translated ORFs within the *S. mutans* contigs. Originally these translated ORFs were used to search for homologues in the SWISS-PROT and TrEMBL databases, and where matches were obtained, a putative function was assigned and the proteins categorized according to their function. However, the publication of the completely sequenced *S. mutans* UA159 genome sequence in 2002 (Ajdić *et al.*, 2002), allowed protein functions to be confirmed and *S. mutans* gene identifications to be assigned to all proteins that had been identified by performing a blast search with the PMM data against annotated *S. mutans* genome sequence database (<http://www.oralgen.lanl.gov/>) (Table 3.1³).

Thirteen additional proteins that could not be identified by PMM, were subjected to ESI-MS/MS. Only 5 provided peptide amino acid sequences. These sequences matched 4 different proteins in the SWISS-PROT, TrEMBL and unfinished microbial genome databases and as with the MALDI-TOF data have since been updated with *S. mutans* gene identifications and functions (Table 3.2⁴).

Out of a total of 200 different proteins that were identified only 34 proteins matched proteins that had been previously characterised from *S. mutans* prior to the release of the completely annotated *S. mutans* genome (Ajdić *et al.*, 2002) (<http://www.oralgen.lanl.gov/>). Consequently, this study identified 166 novel proteins. Not unexpectedly, the closest homologues of these proteins were usually those from other streptococci, particularly *S. pneumoniae* and *S. pyogenes*.

³ Table 3.1 has been placed at the end of chapter 3 (pp. 81-97).

⁴ Table 3.2 has been placed at the end of chapter 3 (pp. 98).

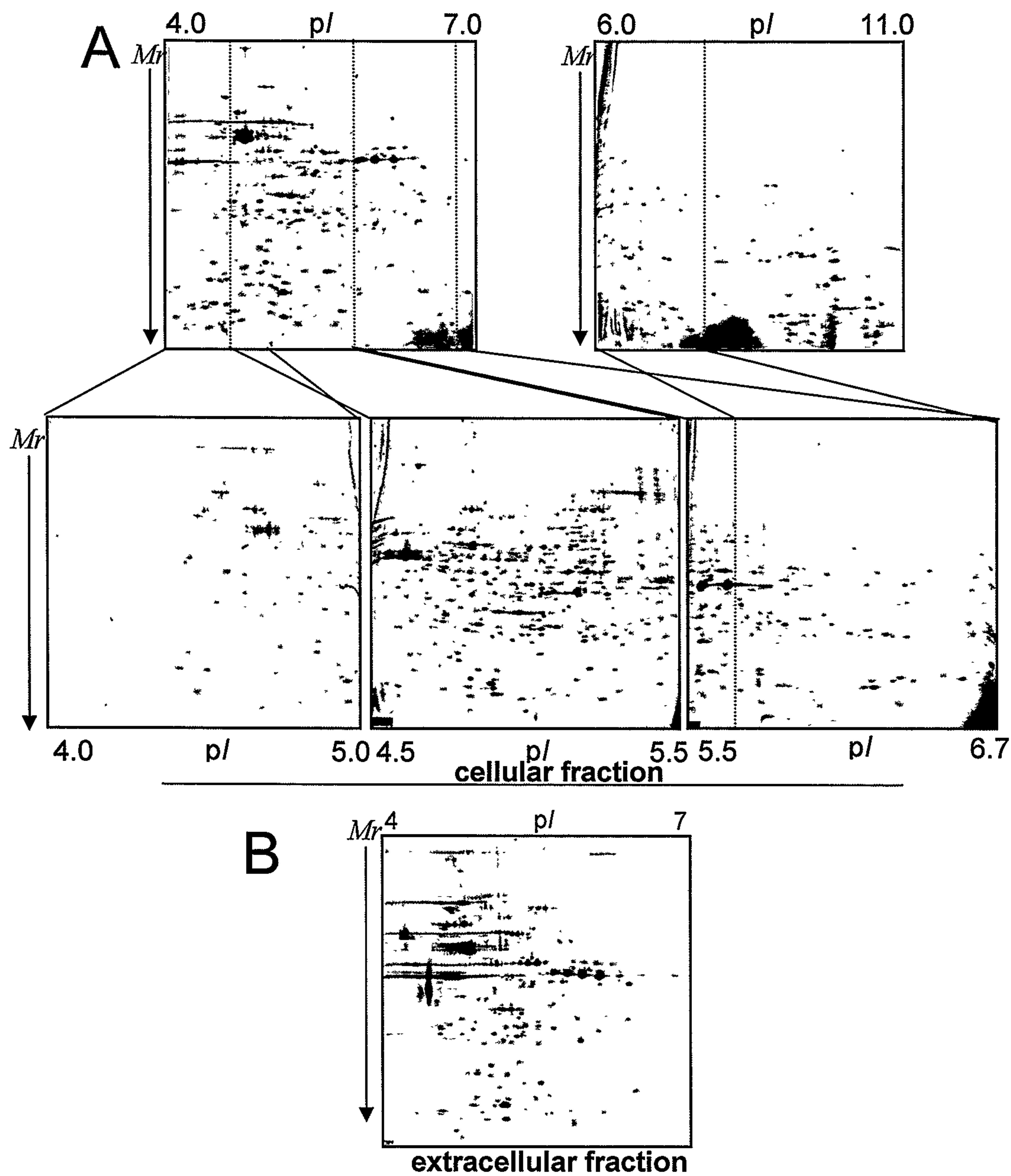


Figure 3.6. 2-DGE proteome maps of *S. mutans* LT11 grown in a glucose limited chemostat at pH 7.0. (A) Soluble cellular proteins were focused in the 1st dimension using mid-range (pH 4.0-7.0, 6.0-11.0) and narrow range (pH 4.5-5.5, 5.5-6.7) IPG-strips. Corresponding 1.0 pH-unit regions are shown in relation to their broad pH range 4.0-7.0 and 6.0-11.0 counterparts. (B) TCA-precipitated proteins from the culture fluid were focused over the pH range 4.0-7.0 prior to separation in the 2nd dimension.

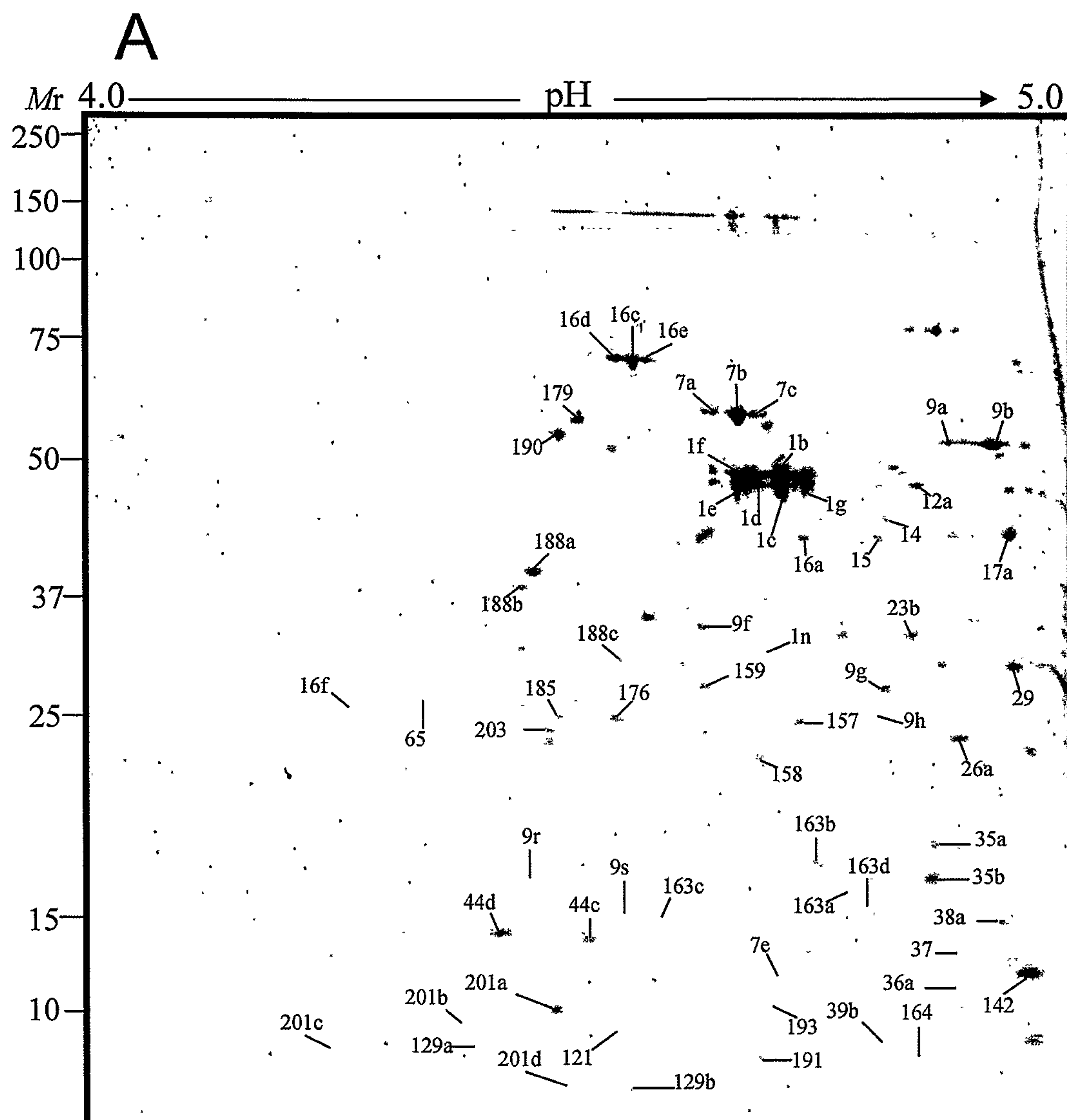
Figure 3.7

Figure 3.7. Solubilized proteins from cells of *S. mutans* LT11 that were identified by MALDI-TOF MS or ESI-MS/MS following separation by 2-DGE. Proteins focused over the pH range 4.0-5.0 (A), 4.5-5.5 (B), 5.5-6.7 (C) and 6.0-11.0 (D) and stained with Sypro Ruby. The numbers refer to the proteins that have been identified (see Table 3.1 and 3.2).

Figure 3.7 (cont)

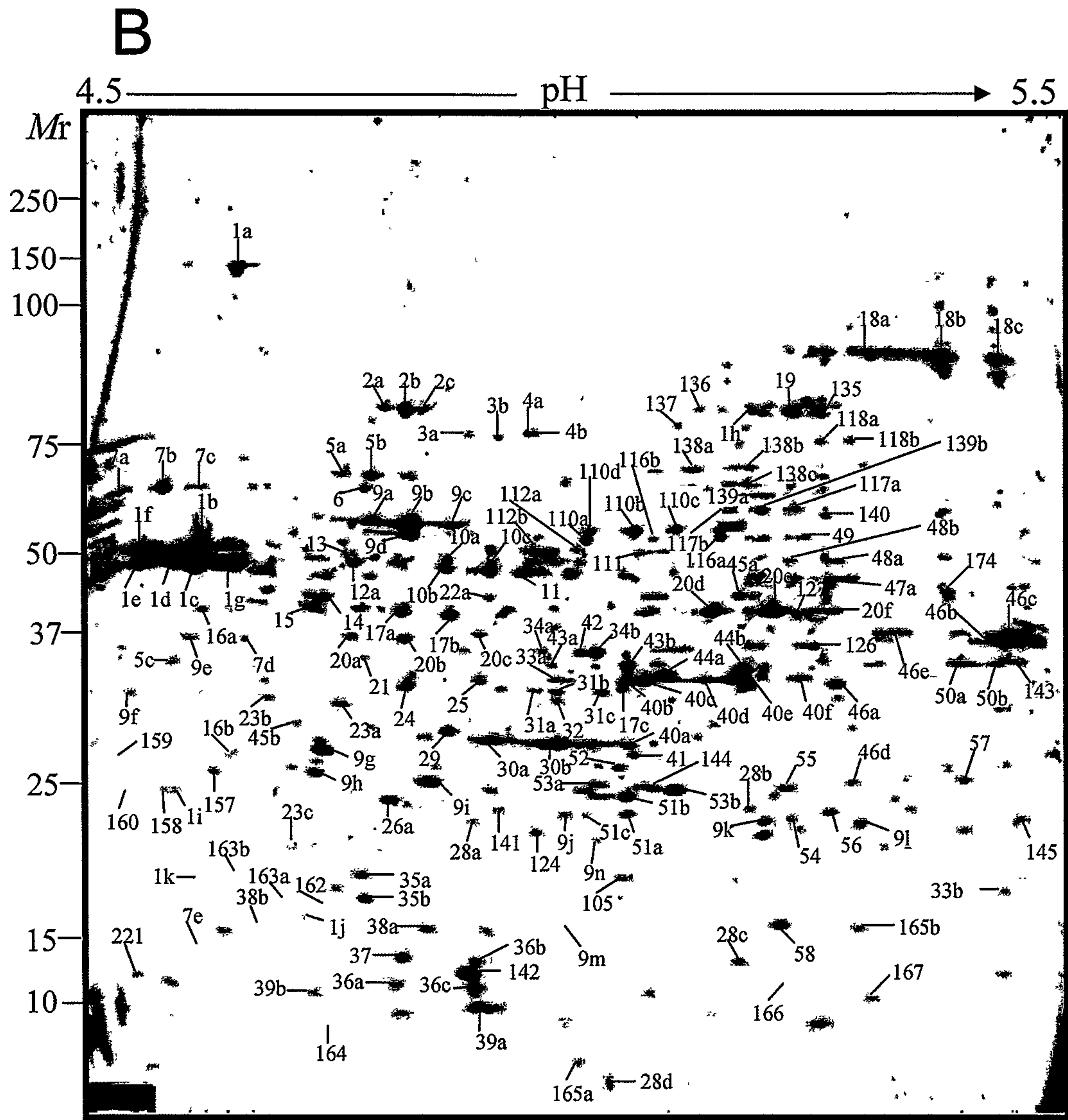


Figure 3.7 (cont)

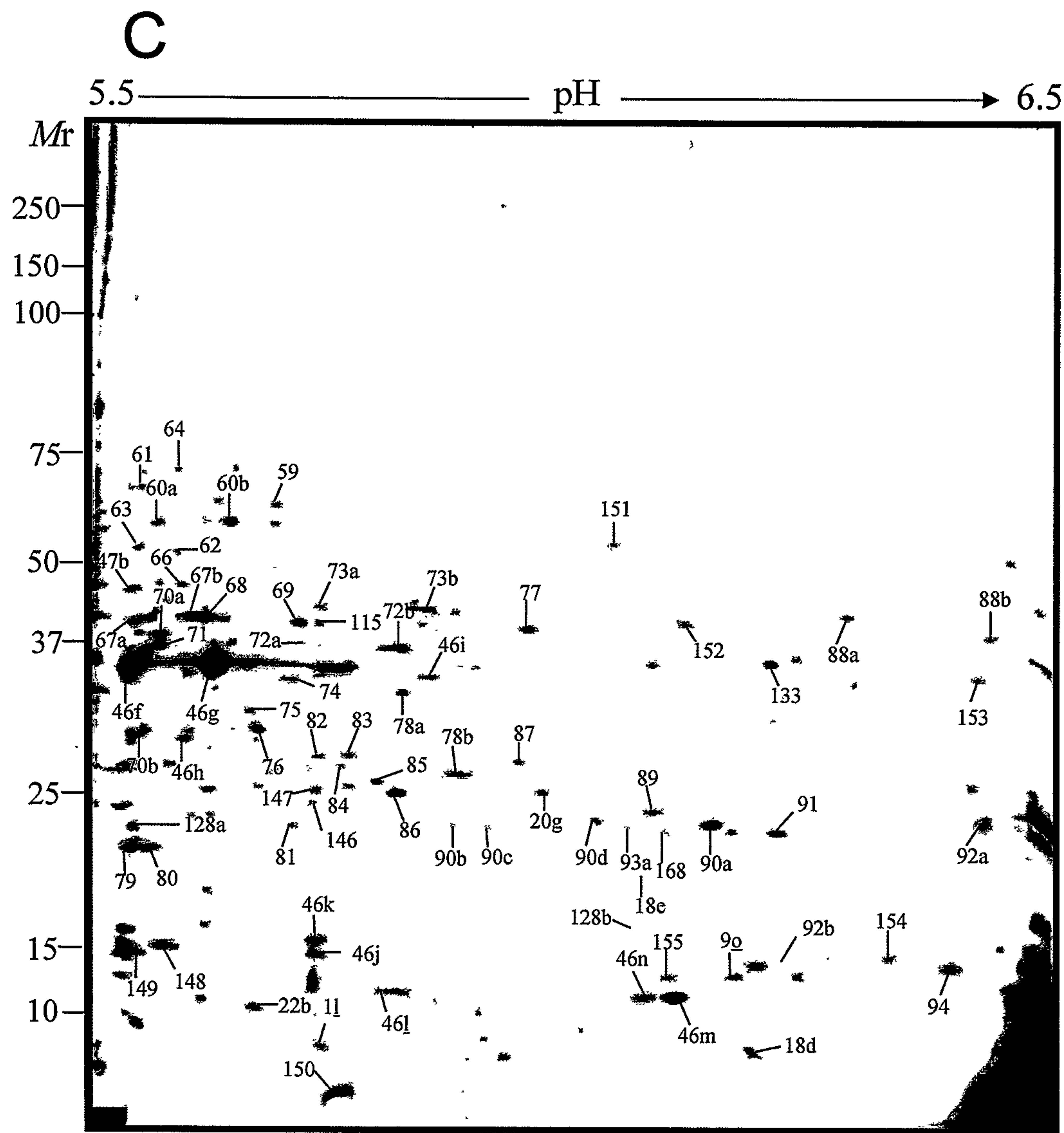


Figure 3.7 (cont)

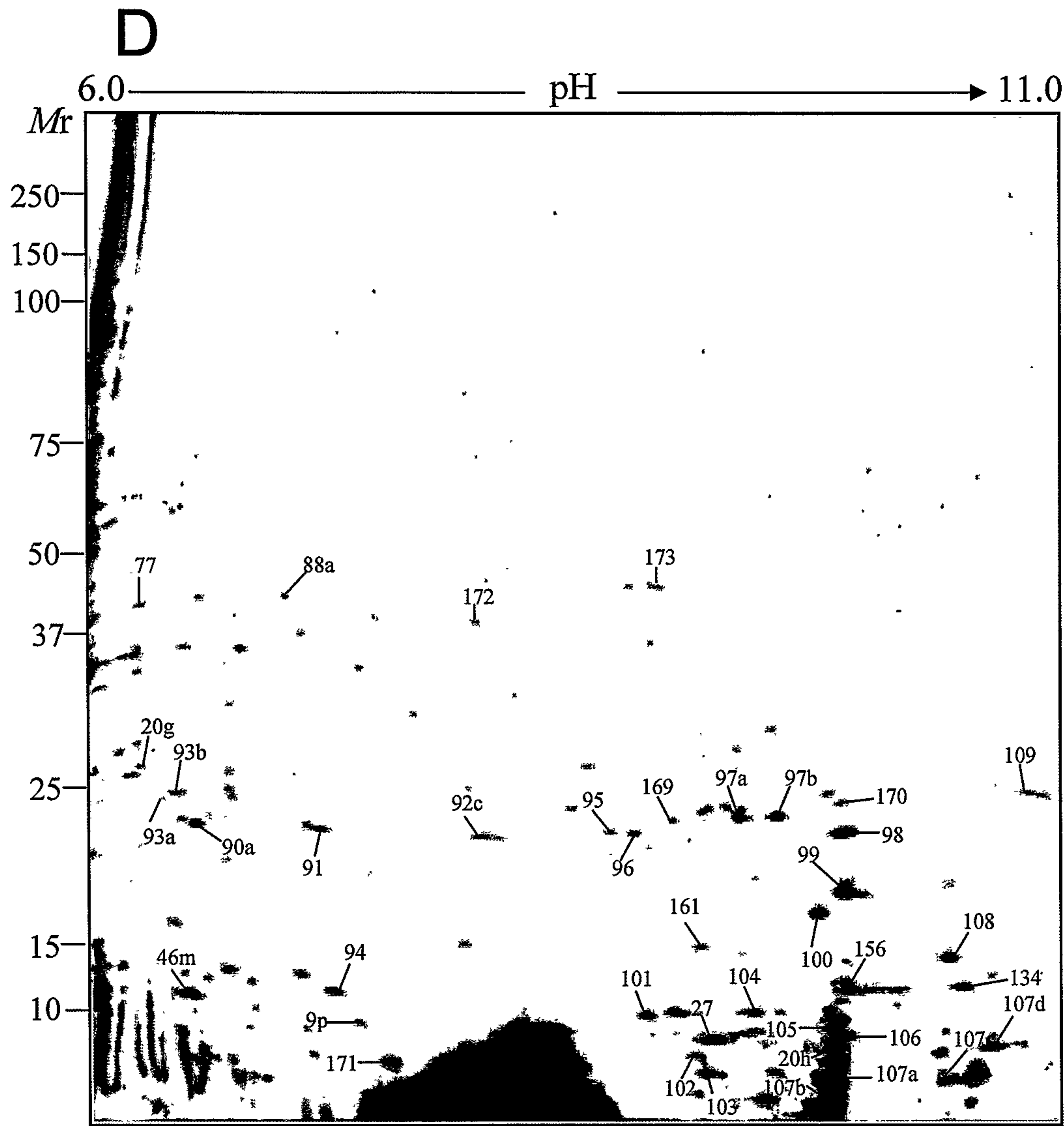


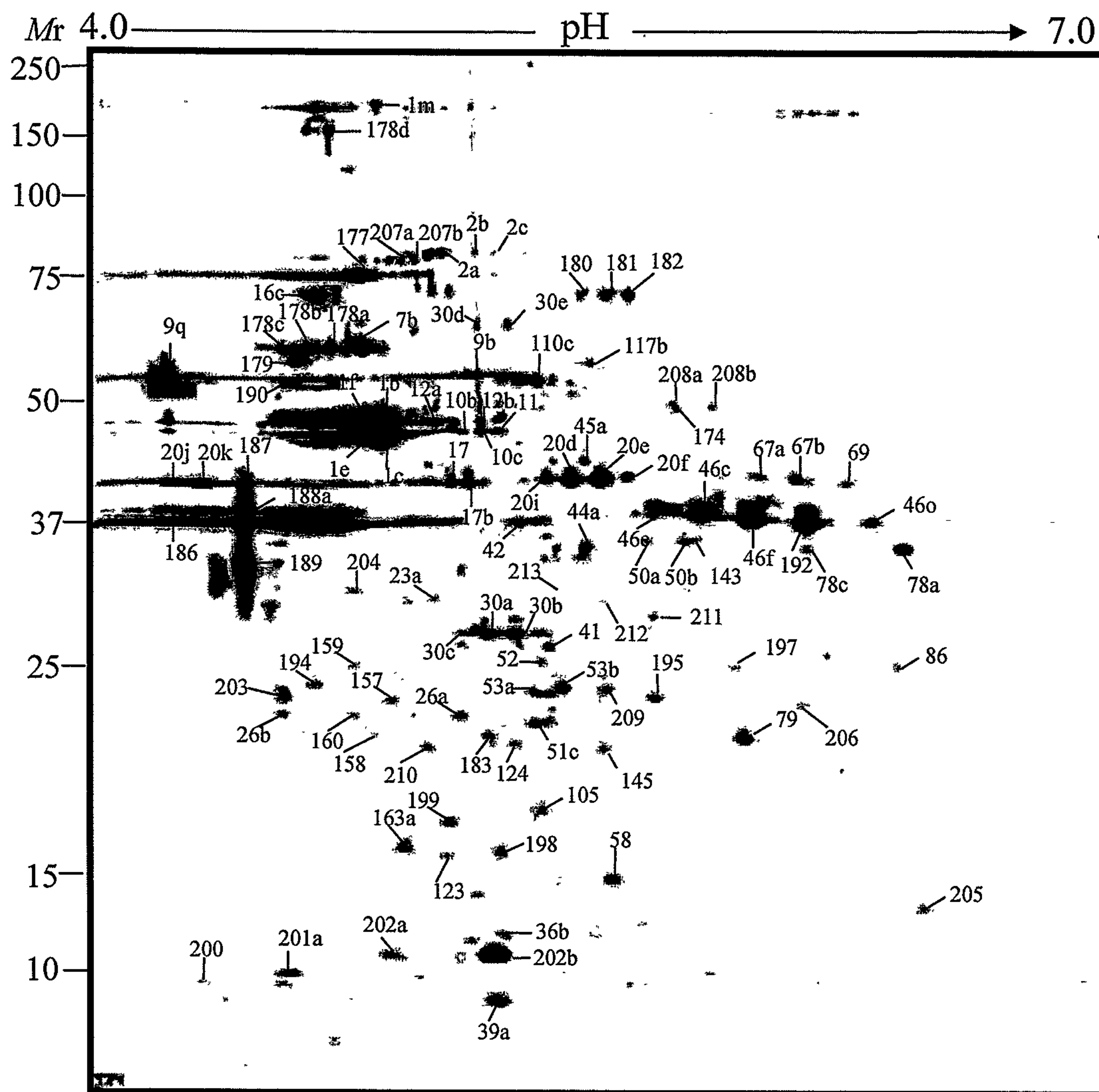
Figure 3.8

Figure 3.8. TCA-precipitated proteins from the culture fluid of chemostat grown *S. mutans* LT11 that were identified by MALDI-TOF MS or ESI-MS/MS following separation by 2-DGE over the pH range 4.0-7.0. The numbers refer to the proteins that have been identified (see Table 3.1 or 3.2).

A comparison of the cellular and culture fluid proteins showed that 15% of those identified (including their modified forms) were found in both compartments. Of the 172 different proteins identified from the cellular fraction, 26 (15%) were functionally undefined. The majority of the identified proteins were associated with house keeping functions such as energy metabolism, amino acid biosynthesis, translation and transport, and binding (Table 3.1).

3.3.3.1 Protein isoforms

Sixty-four proteins detected in either the cellular or extracellular compartments showed isoelectric heterogeneity (charged isoforms) and/or varied in mass. Of the 35 proteins that were detected as pairs of protein spots, 26 were charged isoforms of one another. Isoelectric heterogeneity is considered to be due to post-translational modification of proteins, such as phosphorylation, glycosylation or acylation, or often as the result of an artefact including urea carbamylation or deamidation (Benz and Schmidt, 2002; Klumpp and Krieglstein, 2002; Kussak and Weintraub, 2002; Volkin *et al.*, 1997; Wilkins *et al.*, 1997). The nature of any modification of *S. mutans* proteins observed in this analysis remains to be determined. The 4 most prominently expressed proteins, 3 glycolytic enzymes and the translation elongation factor Tu (SMu0651), however, were detected as more than ten protein spots, many of which were lower *Mr* forms (Table 3.1). Of these, the glycolytic enzyme, enolase (SMu1140), was of particular interest as it formed a cluster of 6 double-banded variants within the *pI* range 4.5 – 4.6 (proteins 1b, c, d, e, f and g; Fig. 3.7B & 3.8), and also existed in two higher mass forms - one approximately three times (protein 1a; Fig. 3.7B) and the other approximately twice the predicted molecular mass and with a more basic *pI* of 5.2 (protein 1h; Fig. 3.7B). Similar multimeric mass variants have been observed in other bacteria including *Staphylococcus aureus* (Cordwell *et al.*, 2002) and *Escherichia coli* (Tonella *et al.*, 1998).

3.3.3.2 Cell wall associated and membrane proteins

Only 3 *S. mutans* wall-associated proteins, WapA, Gbp and SpaP (proteins 189, 177 and 136 respectively; Fig. 3.7B & 3.8) were identified, the former two in the extracellular compartment and the last as an *N*-terminal portion of its *C*-terminally anchored form in the cellular fraction. A

single integral membrane protein was detected in the culture fluid. This was an internal fragment of a homologue of the branched chain amino acid transporter, LivJ (protein 205; Fig. 3.8). Although membrane proteins represent around 30% of total proteins of bacteria (Santoni *et al.*, 2000), membrane-embedded or intrinsic cytoplasmic membrane proteins are rarely, if at all, identified in 2-DGE proteomic maps even in membrane-enriched fractions. This is due to their poor solubility and inherent hydrophobicity. This should be contrasted with membrane-enriched fractions from Gram-negative bacteria which allow the detection of lipoproteins and outer membrane (OM) proteins by 2-DGE (Qi *et al.*, 1996; Langen *et al.*, 2000; Molloy *et al.*, 2000; Nilsson *et al.*, 2000; Nouwens *et al.*, 2000; Molloy *et al.*, 2001; Phadke *et al.*, 2001; Cullen *et al.*, 2002; Hommais *et al.*, 2002). Whereas the transmembrane domains of OM proteins possess β -pleated sheets formed from alternating polar and non-polar amino acids, integral cytoplasmic membrane proteins generally possess hydrophobic α -helical transmembrane domains consisting of non-polar amino acids. Intrinsic membrane proteins therefore, maintain an overall hydropathy that is hydrophobic even when denatured (Santoni *et al.*, 2000). Unfortunately, solubilization of denatured intrinsic cytoplasmic membrane proteins requires the use of surfactants such as SDS which are incompatible with IPG strips. Clearly, different methods of solubilization and/or new methods for selecting or enriching for peptides and proteins associated with cytoplasmic membranes are required for any comprehensive 2-DGE display irrespective of the cell type (Santoni *et al.*, 2000).

3.3.3.3 Extracellular proteins

PMM analysis identified 103 proteins in the culture fluid (Fig. 3.8), 60 of which were also detected in the cytosolic fraction. Of the remaining 43 proteins, 27 are generally considered to be of cellular origin even though they were not detected in the cellular fraction and 4 were 'hypothetical' proteins with no known cellular location (Table 3.1, protein spots 198, 204, 212 and 213). Four additional protein spots present in the extracellular compartment, two of cellular origin and two with unknown function and origin, were identified by peptide ESI-MS/MS (Table 3.8). Consequently, 91 of the 107 proteins identified in the culture fluid would classically be considered to be associated with the cytoplasm or the inner surface of the cytoplasmic membrane, suggesting that they may be the by-products of cell lysis. However, it has recently

become apparent that a number of streptococcal proteins previously thought to be confined to the cytosol are also associated with the cell-surface or secreted into the external milieu (Kolberg and Sletten, 1996; Nelson *et al.*, 2001). For example, in *S. agalactiae*, many of the major surface-bound proteins have glycolytic or chaperonin functions. They include ornithine carbamoyltransferase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, purine nucleoside phosphorylase, enolase, and glucose-6-phosphate isomerase (Hughes *et al.*, 2002). Glyceraldehyde-3-phosphate dehydrogenase is of particular interest as it has been found as a surface-associated protein in every streptococcal species examined so far (Kolberg and Sletten, 1996) and is implicated as a virulence determinant aiding *S. pyogenes* invasion of tissues (Pancholi and Fischetti, 1998; Nelson *et al.*, 2001). In *S. gordonii*, the glyceraldehyde-3-phosphate dehydrogenase, primarily associated with the cell surface at pH 6.5, becomes the major extracellular protein at pH 7.5 (Nelson *et al.*, 2001). This may also be the case for the form of the *S. mutans* enzyme observed in the culture fluid in this study. Other than *S. agalactiae*, surface-bound enolases have been found on a number of streptococci including *S. pyogenes* where the enzyme displays strong plasmin(ogen) binding (Pancholi and Fischetti, 1998; Fontan *et al.*, 2000). How these proteins become associated with the cell-surface without one of the known Gram-positive cell-surface anchoring mechanisms (Cossart and Jonquieres, 2000; Rathsam and Jacques., 1998; Turner *et al.*, 1997), has yet to be determined, though it has been suggested that N-terminal attachment and charge/hydrophobic interactions may be responsible (Fischetti, 2000).

3.4 Conclusion

This initial 2-DGE proteome analysis of *S. mutans* established a comprehensive proteomic reference map of the bacteria. As the map was based on a standardised continuous culture condition, it could be used to compare with other *S. mutans* proteomes derived from modelling environmental conditions relevant to the virulence of the bacteria. Such comparative studies are the subject of the following two chapters

Table 3.1. Proteins identified from *S. mutans* LT11 categorised into putative functional groups

a) spot no. corresponds to spot number on Fig. 3.7 and 3.8. *denotes peptide matching the N-terminus, **denotes peptide matching the C-terminus, ***denotes peptide matching the internal segment of the matching protein.
b) The number of identified peptides from cellular (C) or extracellular (EC) proteins matching a given translated ORF from the *S. mutans* UA159 genome
c) The percentage coverage of the peptides over the matching translated ORF from the *S. mutans* UA159 genome
d) Figure(s) containing the corresponding protein spot

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
I - AMINO ACID BIOSYNTHESIS							
1 Aromatic amino acid family							
87	SMu0706	Shikimate 5-dehydrogenase (<i>aroE</i>)	9		41		3.7C
126	SMu1672	Phospho-2-dehydro-3-deoxyheptonate aldolase (<i>aroG</i>)	7		30		3.7B
151	SMu0709	Prephenate dehydrogenase (<i>tyrA</i>)	4		15		3.7C
152	SMu0708	Chorismate synthase(<i>aroC</i>)	8		31		3.7C
2 Aspartate family							
41	SMu0821	Dihydrodipicolinate reductase (<i>dapB</i>)	8	9	43	44	3.7B, 3.8
49	SMu1430	S-adenosylmethionine synthetase (<i>metK</i>)	10		28		3.7B
127	SMu0899	Aspartate-semialdehyde dehydrogenase (<i>asd</i>)	7		34		3.7B
147	SMu1485	MTA/SAH nucleosidase (<i>pfs</i>)	5		36		3.7C
208-a	SMu0063	Threonine synthase (<i>thrC</i>)		8		20	3.8
208-b	SMu0063	Threonine synthase (<i>thrC</i>)		8		19	3.8
3 Branched chain amino acid family							
21	SMu1297	Acetoin dehydrogenase e1 component (tpp-dependent beta subunit) (<i>acoB</i>)	9		35		3.7B
34-a	SMu0211	Ketol-acid reductoisomerase (<i>ilvC</i>)	7		30		3.7B
34-b	SMu0211	Ketol-acid reductoisomerase (<i>ilvC</i>)	8		34		3.7B
42	SMu1099	Branched-chain amino acid aminotransferase (<i>ilvE</i>)	8	11	30	40	3.7B, 3.8
4 Glutamate family							
174	SMu0829	NADP-Specific glutamate dehydrogenase (<i>gdhA</i>)	10	9	31	20	3.7B, 3.8
5 Serine family							
45-a	SMu1508	Phosphoserine aminotransferase (<i>serC</i>)	8	11	34	32	3.7B, 3.8
45-b**	SMu1508	Phosphoserine aminotransferase (<i>serC</i>)	3		14		3.7B

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
70-a	SMu1505	Phosphoglycerate dehydrogenase (<i>serA</i>)	8		28		3.7C
70-b	SMu1505	Phosphoglycerate dehydrogenase (<i>serA</i>)	8		29		3.7C
78-a	SMu0449	O-acetylserine lyase (<i>cysK</i>)	8	15	46	62	3.7C, 3.8
78-b	SMu0449	O-acetylserine lyase (<i>cysK</i>)	10		55		3.7C
78-c	SMu0449	O-acetylserine lyase (<i>cysK</i>)		11		47	3.8
II - CELL ENVELOPE							
1 Murein sacculus and peptidoglycan							
66	SMu1577	Udp-n-acetyl muramate-alanine ligase (<i>murC</i>)	12		45		3.7C
140	SMu1541	D-Alanine-d-alanyl carrier protein ligase (<i>dltA</i>)	11		28		3.7B
150***	SMu0565	deacetylase (<i>yheN</i>)	5		20		3.7C
195	SMu0578	N-acetylglucosamine-6-phosphate isomerase (<i>nagB</i>)		9		50	3.8
2 Surface antigens							
136*	SMu0556	Cell surface antigen I/II precursor (<i>spaP</i>)	7		6		3.7B
177	SMu0556	Glucan-binding protein precursor (<i>gbp</i>)		11		31	3.8
189	SMu0897	Chain 1: Wall-associated protein (<i>wapA</i>)		5		23	3.8
III - CELLULAR PROCESSES							
1 Cell division							
111	SMu0501	Cell division protein (<i>ftsA</i>)	13		43		3.7B
162**	SMu1375	Chromosome segregation protein (<i>smc</i>)	4		5		3.7B
170	SMu1210	Cell-division ATP-binding protein (<i>ftsE</i>)	9		42		3.7D
188-a	SMu0507	Cell-division initiation protein (septum placement) (<i>divIVA</i>)	10	11	48	41	3.7A, 3.8
188-b	SMu0507	Cell-division initiation protein (septum placement) (<i>divIVA</i>)	6		38		3.7A
188-c	SMu0507	Cell-division initiation protein (septum placement) (<i>divIVA</i>)	5		35		3.7A
190	SMu0502	Cell division protein (<i>ftsZ</i>)	13	10	46	30	3.7A, 3.8
2 Chaperones							
7-a	SMu1774	60 kDa chaperonin (<i>groEL</i>)	18		42		3.7A, 3.7B
7-b	SMu1774	60 kDa chaperonin (<i>groEL</i>)	25	16	55	36	3.7A, 3.7B
7-c	SMu1774	60 kDa chaperonin (<i>groEL</i>)	15		38		3.7A, 3.7B
7-d*	SMu1774	60 kDa chaperonin (<i>groEL</i>)	8		21		3.7B

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
7-e ^{***}	SMu1774	60 kDa chaperonin (<i>groEL</i>)	5		13		3.7A, 3.7B
16-a [*]	SMu0074	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	7		15		3.7A, 3.7B
16-b [*]	SMu0074	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	9		21		3.7B
16-c	SMu0074	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	22	16	52	30	3.7A, 3.8
16-d	SMu0074	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	14		28		3.7A
16-e	SMu0074	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	9		25		3.7A
16-f ^{**}	SMu0074	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	4		13		3.7A
36-a	SMu1775	10 kDa chaperonin (<i>groES</i>)	4		38		3.7A,3.7B
36-b	SMu1775	10 kDa chaperonin (<i>groES</i>)	4	6	38	47	3.7B, 3.8
36-c	SMu1775	10 kDa chaperonin (<i>groES</i>)	6		75		3.7B
179	SMu0082	Trigger factor (<i>ropA</i>)	15	11	45	42	3.7A, 3.8
3 Detoxification							
124	SMu0571	Superoxide dismutase [Mn/Fe] (<i>sodA</i>)	6	5	40	30	3.7B, 3.8
4 Protein and peptide secretion							
142 ^{**}	SMu1513	Signal peptidase II (<i>yaaT</i>)	4		16		3.7A, 3.7B
5 transformation							
154	SMu1684	Late competence protein required for DNA binding (<i>comEB</i>)	4		32		3.7C
IV - CENTRAL INTERMEDIARY METABOLISM							
1 General							
68	SMu1398	Required for glycogen biosynthesis (<i>glgD</i>)	12		40		3.7C
2 Amino acids							
138-a	SMu1086	L-glutamine-D-fructose-6-phosphate amidotransferase (<i>glmS</i>)	10		22		3.7B
138-b	SMu1086	L-glutamine-D-fructose-6-phosphate amidotransferase (<i>glmS</i>)	15		34		3.7B
138-c	SMu1086	L-glutamine-D-fructose-6-phosphate amidotransferase (<i>glmS</i>)	5		13		3.7B
V- ENERGY METABOLISM							
1 Aerobic							

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
56	SMu1135	Nitroreductase family protein (<i>nrd</i>)	6		41		3.7B
96	SMu1457	NAD(P)H-flavin oxidoreductase (<i>yfkO</i>)	6		25		3.7D
74	SMu0290	Glycerol-3-phosphate dehydrogenase (<i>gpdA</i>)	7		33		3.7C
2 ATP-proton motive force interconversions							
155	SMu1388	ATP synthase epsilon chain (<i>atpC</i>)	4		44		3.7C
3 Fatty acid and phospholipid metabolism							
67-a	SMu1585	3-oxoacyl-(acyl-carrier-protein) synthase (<i>fabF</i>)	6	7	28	27	3.7C, 3.8
67-b	SMu1585	3-oxoacyl-(acyl-carrier-protein) synthase (<i>fabF</i>)	13	7	54	28	3.7C, 3.8
210**	SMu0061	Acyltransferase (<i>spy0041</i>)		10		19	3.8
4 Fermentation							
18-a	SMu0131	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	7		12		3.7B
18-b	SMu0131	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	5		10		3.7B
18-c	SMu0131	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	9		16		3.7B
18-d***	SMu0131	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	4		6		3.7C
18-e***	SMu0131	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	4		8		3.7C
25	SMu0952	Phosphotransacetylase (<i>pta</i>)	8		31		3.7B
69	SMu1796	Acetate kinase (<i>ackA</i>)	12	10	41	30	3.7C, 3.8
85	SMu1207	Acetoin reductase (<i>butA</i>)	8		52		3.7C
135	SMu0363	Formate acetyltransferase (<i>pfl</i>)	13		21		3.7B
91	SMu1516	acetoin utilization protein, acetoin dehydrogenase (<i>acuB</i>)	9		62		3.7C, 3.7D
5 Glycogen synthesis							

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
56	SMu1135	Nitroreductase family protein (<i>nrd</i>)	6		41		3.7B
6 Glycolysis							
1-a	SMu1140	Enolase (<i>eno</i>)	15		48		3.7B
1-b	SMu1140	Enolase (<i>eno</i>)	18	10	52	30	3.7B, 3.8
1-c	SMu1140	Enolase (<i>eno</i>)	18	9	56	29	3.7B, 3.8
1-d	SMu1140	Enolase (<i>eno</i>)	14		50		3.7B
1-e	SMu1140	Enolase (<i>eno</i>)	12	9	45	27	3.7B, 3.8
1-f	SMu1140	Enolase (<i>eno</i>)	13	14	47	47	3.7B, 3.8
1-g	SMu1140	Enolase (<i>eno</i>)	12		46		3.7B
1-h	SMu1140	Enolase (<i>eno</i>)	11		41		3.7B
1-i**	SMu1140	Enolase (<i>eno</i>)	5		15		3.7B
1-j**	SMu1140	Enolase (<i>eno</i>)	5		18		3.7B
1-k*	SMu1140	Enolase (<i>eno</i>)	4		17		3.7B
1-l*	SMu1140	Enolase (<i>eno</i>)	3		10		3.7C
1-m	SMu1140	Enolase (<i>eno</i>)		13		35	3.8
1-n*	SMu1140	Enolase (<i>eno</i>)	3		15		3.7A
5-a	SMu0985	Phosphoglucomutase (<i>pgmA</i>)	15		40		3.7B
5-b	SMu0985	Phosphoglucomutase (<i>pgmA</i>)	25		63		3.7B
5-c*	SMu0985	Phosphoglucomutase (<i>pgmA</i>)	7		18		3.7B
12-a	SMu0277	Glucose-6-phosphate isomerase (<i>gpi</i>)	17	17	50	45	3.7B, 3.8
12-b	SMu0277	Glucose-6-phosphate isomerase (<i>gpi</i>)		6		13	3.8
20-a	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	10		32		3.7B
20-b	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	14		46		3.7B
20-c	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	8		25		3.7B
20-d	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	18	6	53	19	3.7B, 3.8
20-e	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	16	6	51	19	3.7B, 3.8
20-f	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	8	6	25	23	3.7B, 3.8
20-g	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	8		25		3.7C, 3.7D
20-h	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)	5		13		3.7D
20-I	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)		16		44	3.8
20-j	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)		13		33	3.8
20-k	SMu0326	Phosphoglycerate kinase (<i>pgk</i>)		21		66	3.8
30-a	SMu0088	Class-II aldolase (<i>fba</i>)	11	6	48	41	3.7B, 3.8

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
30-b	SMu0088	Class-II aldolase (<i>fba</i>)	10	8	38	30	3.7B, 3.8
30-c	SMu0088	Class-II aldolase (<i>fba</i>)		7		41	3.8
30-d	SMu0088	Class-II aldolase (<i>fba</i>)		7		33	3.8
30-e	SMu0088	Class-II aldolase (<i>fba</i>)		9		43	3.8
43-a	SMu1018	L-lactate dehydrogenase (<i>ldh</i>)	6		26		3.7B
43-b	SMu1018	L-lactate dehydrogenase (<i>ldh</i>)	9		31		3.7B
46-a	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	11		39		3.7B
46-b	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	9		23		3.7B
46-c	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	11	10	29	27	3.7B, 3.8
46-d**	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	5		20		3.7B
46-e	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	10	13	36	37	3.7B, 3.8
46-f	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	12	10	40	27	3.7C, 3.8
46-g	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	17		43		3.7C
46-h	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	10		32		3.7C
46-I	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	6		16		3.7C
46-j*	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	5		14		3.7C
46-k*	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	4		10		3.7C
46-l*	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	6		19		3.7C
46-m**	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	7		22		3.7C, 3.7D
46-n*	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)	4		12		3.7C

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
46-o*	SMu0325	Glyceraldehyde 3-phosphate dehydrogenase (<i>gapDH</i>)		7		22	3.8
50-a	SMu1090	6-phosphofructokinase (<i>pfkA</i>)	7	7	23	18	3.7B, 3.8
50-b	SMu1090	6-phosphofructokinase (<i>pfkA</i>)	9	7	34	18	3.7B, 3.8
51-a	SMu0652	Triosephosphate isomerase (<i>tpiA</i>)	8		44		3.7B
51-b	SMu0652	Triosephosphate isomerase (<i>tpiA</i>)	5		33		3.7B
51-c	SMu0652	Triosephosphate isomerase (<i>tpiA</i>)	7	7	44	44	3.7B, 3.8
53-a	SMu0543	Phosphoglyceromutase (<i>gpmA</i>)	4	8	21	32	3.7B, 3.8
53-b	SMu0543	Phosphoglyceromutase (<i>gpmA</i>)	11	12	42	56	3.7B, 3.8
110-a	SMu1089	Pyruvate kinase (<i>pyk</i>)	14		40		3.7B
110-b	SMu1089	Pyruvate kinase (<i>pyk</i>)	12		28		3.7B
110-c	SMu1089	Pyruvate kinase (<i>pyk</i>)	12	11	33	22	3.7B, 3.8
110-d	SMu1089	Pyruvate kinase (<i>pyk</i>)	18		48		3.7B
7 Pentose phosphate pathway							
4-a	SMu0262	Transketolase (<i>tkt</i>)	8		17		3.7B
4-b	SMu0262	Transketolase (<i>tkt</i>)	13		26		3.7B
157	SMu1129	Ribose 5-phosphate isomerase (<i>rpiA</i>)	11	8	51	43	3.7B, 3.8
8 Pyruvate dehydrogenase							
62	SMu1299	Dihydrolipoamide dehydrogenase, component E3 (<i>acoL</i>)	6		26		3.7C
9 Sugars							
24	SMu0493	Glucose kinase (<i>glcK</i>)	9		35		3.7B
61	SMu1423	4-alpha-glucanotransferase (<i>malM</i>)	5		15		3.7C
143	SMu0810	UDP-glucose 4-epimerase (<i>galE</i>)	9	8	35	31	3.7B, 3.8
211	SMu1676	Fructokinase (<i>scrK</i>)		4		21	3.8
10 TCA cycle							
11	SMu1930	Succinic semialdehyde dehydrogenase (<i>gabD</i>)	13	6	31	14	3.7B, 3.8

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
160*	SMu0285	2,3,4,5-Tetrahydropyridine-2-carboxylate N-succinyltransferase-related protein (<i>dapD</i>)	5	8	33	43	3.7B, 3.8
VI FATTY ACID METABOLISM							
76	SMu1588	Trans-2-enoyl-acp reductase II (<i>sabK</i>)	9		46		3.7C
92-a	SMu1586	Beta-ketoacyl-acp reductase (<i>fabG</i>)	7		44		3.7C
92-b*	SMu1586	Beta-ketoacyl-acp reductase (<i>fabG</i>)	9		42		3.7C
92-c	SMu1586	Beta-ketoacyl-acp reductase (<i>fabG</i>)	9		45		3.7D
101	SMu1583	(3R)-Hydroxymyristoyl-[acyl carrier protein] dehydratase (<i>fabZ</i>)	6		35		3.7D
VII - PURINES, PYRAMIDINES, NUCLEOSIDES AND NUCLEOTIDES							
1 Purine ribonucleotide biosynthesis							
60-a	SMu1957	Inosine-5'-monophosphate dehydrogenase (<i>guaB</i>)	11		29		3.7C
60-b	SMu1957	Inosine-5'-monophosphate dehydrogenase (<i>guaB</i>)	14		34		3.7C
2 Pyrimidine ribonucleotide biosynthesis							
57	SMu1479	Uridylate kinase (<i>pyrH</i>)	6		37		3.7B
3 Salvage of nucleotides and nucleosides							
141	SMu0432	Guanylate kinase (<i>gmk</i>)	8		36		3.7B
168	SMu1525	Uracil phosphoribosyl-transferase (<i>upp</i>)	8		56		3.7C
206	SMu1026	Deoxyribose-phosphate aldolase (<i>deoC</i>)		4		24	3.8
209	SMu1821	Adenylate kinase (<i>adk</i>)		7		42	3.8
4 Sugar-nucleotide biosynthesis and interconversions							
29	SMu1330	Glucose-1-phosphate thymidyltransferase (<i>rmlA</i>)	15		52		3.7A, 3.7B
32	SMu0748	DTDP-4-keto-L-rhamnose reductase (<i>rmlD</i>)	6		27		3.7B
71	SMu1327	DTDP-Glucose 4,6-dehydratase (<i>rmlB</i>)	9		32		3.7C

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
183	SMu1329	DTDP-4-keto-L-rhamnose reductase (<i>rmlC</i>)		8		50	3.8
VIII - REGULATORY FUNCTIONS							
1 General							
37	SMu1195	Possible AldB regulatory protein	3		28		3.7A, 3.7B
55	SMu1748	response regulator GcrR for glucan-binding protein(<i>gcrR</i>)	7		44		3.7B
28-a	SMu0373	Cell-cycle regulation histidine triad protein (<i>hit</i>)	4		31		3.7B
28-b	SMu0373	Cell-cycle regulation histidine triad protein (<i>hit</i>)	4		30		3.7B
28-c	SMu0373	Cell-cycle regulation histidine triad protein (<i>hit</i>)	8		75		3.7B
28-d*	SMu0373	Cell-cycle regulation histidine triad protein (<i>hit</i>)	3		30		3.7B
185	SMu0437	Phosphoprotein phosphatase (<i>pppL</i>)	3		19		3.7A
2 Lac-I family regulators							
133	SMu1446	Catabolite control protein A (<i>ccpA</i>)	9		40		3.7C
IX - REPLICATION							
1 DNA replication, restriction, modification, recombination and repair							
13	SMu1514	DNA polymerase III delta prime subunit (<i>holB</i>)	9		27		3.7B
47-a	SMu1892	Recombinase A (<i>recA</i>)	9		35		3.7B
47-b	SMu1892	Recombinase A (<i>recA</i>)	8		31		3.7C
107-a	SMu0536	histone-like DNA-binding protein(<i>hlpA</i>)	5		50		3.7D
107-b	SMu0536	histone-like DNA-binding protein(<i>hlpA</i>)	5		50		3.7D
107-c	SMu0536	histone-like DNA-binding protein(<i>hlpA</i>)	7		67		3.7D
107-d	SMu0536	histone-like DNA-binding protein(<i>hlpA</i>)	4		45		3.7D
165-a*	SMu1325	Mutator mutt protein (<i>mutX</i>)	3		21		3.7B
165-b	SMu1325	Mutator mutt protein (<i>mutX</i>)	4		30		3.7B
181	SMu1746	Replication protein (<i>dnaB</i>)		8		24	3.8
2 Degradation of DNA							
75	SMu1501	exodeoxyribonuclease III (<i>exoA</i>)	7		37		3.7C
207-a	SMu1108	5'-nucleotidase precursor		11		26	3.8
207-b	SMu1108	5'-nucleotidase precursor		7		15	3.8

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
1 Degradation of RNA							
115**	SMu0138	Polyribonucleotide nucleotidyl-transferase (<i>pnpA</i>)	6		13		3.7C
2 RNA synthesis, modification, and DNA transcription							
6	SMu0379	Transcription termination-antitermination factor (<i>nusA</i>)	13		39		3.7B
15	SMu1817	DNA-Directed RNA polymerase alpha subunit (<i>rpoA</i>)	18		58		3.7B
35-a	SMu1574	Transcription elongation factor (<i>greA</i>)	7		63		3.7B
35-b	SMu1574	Transcription elongation factor (<i>greA</i>)	10		64		3.7B
199	SMu1574	Transcription elongation factor (<i>greA</i>)		5		30	3.8
153	SMu0304	RNA-binding protein, Jag family	8		26		3.7C
XI - TRANSLATION							
1 Aminoacyl tRNA synthetases							
59	SMu1904	Arginyl-tRNA synthetase (<i>argS</i>)	17		36		3.7C
72-a	SMu1374	Phenylalanyl-tRNA synthetase (alpha subunit) (<i>pheS</i>)	7		24		3.7C
72-b	SMu1374	Phenylalanyl-tRNA synthetase (alpha subunit) (<i>pheS</i>)	6		22		3.7C
88-a	SMu1809	Tyrosyl-tRNA synthetase 1 (<i>tyrS</i>)	8		28		3.7C
88-b	SMu1809	Tyrosyl-tRNA synthetase 1 (<i>tyrS</i>)	8		22		3.7C
112-a	SMu1657	Glutamyl tRNA-gln amido-transferase subunit B (<i>gatB</i>)	12		34		3.7B
112-b	SMu1657	Glutamyl tRNA-gln amido-transferase subunit B (<i>gatB</i>)	10		31		3.7B
116-a	SMu1658	Glutamyl tRNA-gln amido-transferase subunit A (<i>gatA</i>)	15		36		3.7B
116-b	SMu1658	Glutamyl tRNA-gln amido-transferase subunit A (<i>gatA</i>)	10		28		3.7B
117-a	SMu0296	Glutamyl-tRNA synthetase (<i>gltX</i>)	10		22		3.7B
117-b	SMu0296	Glutamyl-tRNA synthetase (<i>gltX</i>)	17	14	42	37	3.7B, 3.8

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
118-a	SMu1441	Threonyl-tRNA synthetase 1 (<i>thrS</i>)	12		23		3.7B
118-b	SMu1441	Threonyl-tRNA synthetase 1 (<i>thrS</i>)	11		22		3.7B
137	SMu1492	Methionyl-tRNA synthetase (<i>metS</i>)	10		20		3.7B
139-a	SMu0701	Lysyl-tRNA synthetase (<i>lysS</i>)	13		29		3.7B
139-b	SMu0701	Lysyl-tRNA synthetase (<i>lysS</i>)	13		29		3.7B
200	SMu1659	Glutamyl tRNA-gln amido-transferase subunit C (<i>gatC</i>)		6		58	3.8
2 Degradation of proteins, peptides and glycoproteins							
48-a	SMu0423	Aminopeptidase C (<i>pepC</i>)	9		26		3.7B
48-b	SMu0423	Aminopeptidase C (<i>pepC</i>)	11		34		3.7B
145	SMu1524	ATP-Dependent clp protease proteolytic subunit (<i>clpP</i>)	3	5	18	19	3.7B, 3.8
182	SMu0691	Protease (<i>yugD</i>)		8		29	3.8
3 Protein modifications							
128-a	SMu0128	Polypeptide deformylase (<i>def</i>)	6		43		3.7C
128-b*	SMu0128	Polypeptide deformylase (<i>def</i>)	3		21		3.7C
4 Ribosomal proteins: synthesis and modifications							
10-a	SMu1097	Ribosomal protein S1-like DNA-binding protein (<i>rpsA</i>)	7		32		3.7B
10-b	SMu1097	Ribosomal protein S1-like DNA-binding protein (<i>rpsA</i>)	15	9	44	23	3.7B, 3.8
10-c	SMu1097	Ribosomal protein S1-like DNA-binding protein (<i>rpsA</i>)	12	13	35	33	3.7B, 3.8
31-a	SMu1847	30S Ribosomal protein S2 (<i>rpsB</i>)	7		29		3.7B
31-b	SMu1847	30S Ribosomal protein S2 (<i>rpsB</i>)	8		34		3.7B
31-c	SMu1847	30S Ribosomal protein S2 (<i>rpsB</i>)	8		35		3.7B
39-a	SMu1694	30S Ribosomal protein S6 (<i>rpsF</i>)	6	5	74	67	3.7B, 3.8
39-b	SMu1694	30S Ribosomal protein S6 (<i>rpsF</i>)	4		67		3.7A, 3.7B
97-a	SMu1480	50S ribosomal protein L1 (<i>rplA</i>)	6		33		3.7D
97-b	SMu1480	50S ribosomal protein L1 (<i>rplA</i>)	5		31		3.7D
98	SMu1836	30S Ribosomal protein S3(<i>rpsC</i>)	6		41		3.7D

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
99	SMu1827	50S Ribosomal protein L6(<i>rplF</i>)	5		29		3.7D
100	SMu1830	50S Ribosomal protein L5(<i>rplE</i>)	9		58		3.7D
103	SMu1185	50S Ribosomal protein L31 type B (<i>rpmE2</i>)	6		67		3.7D
104	SMu1828	30S Ribosomal protein S8 (<i>rpsH</i>)	5		52		3.7D
106	SMu0869	50S Ribosomal protein L21 (<i>rplU</i>)	3		49		3.7D
108	SMu0152	50S Ribosomal protein L13 (<i>rplM</i>)	3		39		3.7D
109	SMu1840	50S Ribosomal protein L3 (<i>rplC</i>)	3		22		3.7D
123	SMu0869	50S Ribosomal protein L10 (<i>rplJ</i>)		7		39	3.8
134	SMu1816	50S Ribosomal protein L17 (<i>rplQ</i>)	3		42		3.7D
156	SMu1941	50S Ribosomal protein L9 (<i>rplI</i>)	4		19		3.7D
201-a	SMu0872	50S Ribosomal protein L7/L12 (<i>rplL</i>)	6	7	62	64	3.7A, 3.8
201-b	SMu0872	50S Ribosomal protein L7/L12 (<i>rplL</i>)	3		45		3.7A
201-c	SMu0872	50S Ribosomal protein L7/L12 (<i>rplL</i>)	3		45		3.7A
201-d	SMu0872	50S Ribosomal protein L7/L12 (<i>rplL</i>)	4		48		3.7A
90-a	SMu0453	ribosome-associated protein (<i>yfiA</i>)	10		73		3.7C, 3.7D
90-b	SMu0453	ribosome-associated protein (<i>yfiA</i>)	8		71		3.7C
90-c	SMu0453	ribosome-associated protein (<i>yfiA</i>)	8		71		3.7C
90-d	SMu0453	ribosome-associated protein (<i>yfiA</i>)	7		57		3.7C
5 Translation factors							
2-a	SMu0324	Translation elongation factor G (<i>fusA</i>)	13	14	26	26	3.7B, 3.8
2-b	SMu0324	Translation elongation factor G (<i>fusA</i>)	21	7	38	13	3.7B, 3.8
2-c	SMu0324	Translation elongation factor G (<i>fusA</i>)	12	19	24	33	3.7B, 3.8
9-a	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	12		35		3.7A, 3.7B
9-b	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	13	16	43	47	3.7A, 3.7B, 3.8
9-c	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	11		29		3.7B
9-d	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	17		51		3.7B

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
9-e [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	8		20		3.7B
9-f [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	5		15		3.7A, 3.7B
9-g ^{***}	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	8		18		3.7A, 3.7B
9-h ^{**}	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	7		22		3.7A, 3.7B
9-i [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	11		44		3.7B
9-j ^{**}	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	5		15		3.7B
9-k ^{***}	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	12		40		3.7B
9-l [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	8		28		3.7B
9-m [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	4		11		3.7B
9-n ^{**}	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	6		17		3.7B
9-o ^{***}	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	7		21		3.7C
9-p [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	7		17		3.7D
9-q	SMu0651	Translation elongation factor Tu (<i>tuf</i>)		10		31	3.8
9-r [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	5		17		3.7A
9-s [*]	SMu0651	Translation elongation factor Tu (<i>tuf</i>)	4		12		3.7A
17-a	SMu1846	Translation elongation factor TS (<i>tsf</i>)	6	14	30	58	3.7A, 3.7B, 3.8
17-b	SMu1846	Translation elongation factor TS (<i>tsf</i>)	20	14	78	56	3.7B, 3.8
17-c ^{**}	SMu1846	Translation elongation factor TS (<i>tsf</i>)	5		22		3.7B
22-a	SMu0993	Peptide chain release factor 1 (<i>prfA</i>)	7		27		3.7B
22-b ^{***}	SMu0993	Peptide chain release factor 1 (<i>prfA</i>)	3		8		3.7C
26-a	SMu1682	Translation elongation factor P (<i>elp</i>)	5	5	42	31	3.7A, 3.7B, 3.8
26-b	SMu1682	Translation elongation factor P (<i>elp</i>)		4		26	3.8
79	SMu1478	Ribosome recycling factor (<i>ffr</i>)	9	4	51	20	3.7C, 3.8
XII - TRANSPORT and BINDING PROTEINS							
1 Amino acids, peptides and amines							
65	SMu0786	ABC transporter, ATP-binding protein	3		34		3.7A

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
81	SMu1517	Branched-chain amino acid ABC transporter, ATP-binding protein (livF)	7		36		3.7C
84	SMu0418	ABC transporter, ATP-binding protein	6		30		3.7C
86	SMu1380	glutamine ABC transport, ATP-binding protein	9	5	57	25	3.7C, 3.8
144	SMu0517	Amino acid ABC transporter, ATP-binding protein (glnQ)	6		37		3.7B
159	SMu0224	ABC transporter ATP binding protein (<i>yurY</i>)	9	5	48	27	3.7A, 3.7B, 3.8
169	SMu0218	Amino acid ABC transporter, ATP-binding protein	5		38		3.7D
205***	SMu1521	ABC transporter, periplasmic branched chain amino acid-binding (<i>livJ</i>)		7		21	3.8
2 Carbohydrates							
73-a	SMu0805	Multiple sugar-binding transport ATP-binding protein (<i>msmK</i>)	7		25		3.7C
73-b	SMu0805	Multiple sugar-binding transport ATP-binding protein (<i>msmK</i>)	6		24		3.7C
77	SMu1428	ABC-type transport system ATP-binding protein (maltose) (<i>msmK</i>)	6		22		3.7C, 3.7D
3 Cations							
23-a	SMu1537	Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	9	7	39	33	3.7B, 3.8
23-b	SMu1537	Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	8		36		3.7A, 3.7B
23-c**	SMu1537	Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	4		16		3.7B
89	SMu0164	ABC Transporter ATP-binding protein-manganese transport (<i>sloA</i>)	8		42		3.7C
93-a	SMu0164	SloA (<i>sloA</i>)	9		50		3.7C, 3.7D
93-b	SMu0164	SloA (<i>sloA</i>)	7		38		3.7D
4 PTS							
44-a	SMu1707	Mannose-specific phospho-transferase system component IIAB (<i>manL</i>)	8	14	33	47	3.7B, 3.8

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
44-b	SMu1707	Mannose-specific phospho-transferase system component IIAB (<i>manL</i>)	11		45		3.7A, 3.7B
44-c*	SMu1707	Mannose-specific phospho-transferase system component IIAB (<i>manL</i>)	6		23		3.7A
5 General							
172	Smu1920	glycine betaine / carnitine / choline ABC transporter, ATP-binding protein, opuCA (<i>opuCA</i>)	9		30		3.7D
1 Adaptation and atypical conditions							
3-a	SMu0868	ATP-Dependent protease ATP-binding subunit (<i>clpL</i>)	20		37		3.7B
3-b	SMu0868	ATP-Dependent protease ATP-binding subunit (<i>clpL</i>)	28		55		3.7B
19	SMu0511	ATP-Dependent protease (<i>clpE</i>)	9		15		3.7B
52	SMu1379	two-component response regulator (<i>covR</i>)	5	8	39	32	3.7B, 3.8
163-a	SMu0491	DPR protein (<i>dpr</i>)	8	7	45	45	3.7A, 3.7B, 3.8
163-b	SMu0491	DPR protein (<i>dpr</i>)	10		52		3.7A, 3.7B
163-c	SMu0491	DPR protein (<i>dpr</i>)	6		35		3.7A
163-d	SMu0491	DPR protein (<i>dpr</i>)	5		36		3.7A
167	SMu0207	Alkaline-shock protein (<i>asp</i>)	3		26		3.7B
176	SMu0073	HSP-70 co-factor (<i>grpE</i>)	5		38		3.7A
178-a	SMu0018	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		7		18	3.8
178-b	SMu0018	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		9		24	3.8
178-c	SMu0018	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		6		18	3.8
178-d	SMu0018	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		8		20	3.8
180	-	Group B <i>streptococcus</i> secreted protein (<i>bsp</i>)		10		23	3.8
186	-	Glyceraldehyde-3-phosphate dehydrogenase [plasminogen-binding protein] (<i>gpd</i>)		14		59	3.8
192	-	Extracellular glyceraldehyde-3-phosphate dehydrogenase [fragment]		9		27	3.8
193	SMu1754	LemA like protein (<i>lemA</i>)	6		36		3.7A
194	SMu0073	GrpE protein [HSP-70 co-factor] (<i>grpE</i>)		4		28	3.8
2 Phage related functions							

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
105	SMu1693	single strand binding protein (<i>ssb</i>)	5	6	39	45	3.7B, 3.8
3 Transposon related functions							
173	SMu0942	Transposase from transposon TN1545 (<i>int</i>)	8		26		3.7D
4 No function assigned							
27	SMu0189	hypothetical protein	4		47		3.7D
33-a	SMu1602	Conserved hypothetical protein	5		24		3.7B
33-b*	SMu1602	Conserved hypothetical protein	3		14		3.7B
38-a	SMu1665	Hypothetical protein	8		81		3.7A, 3.7B
38-b	SMu1665	Hypothetical protein	4		35		3.7B
40-a	SMu1602	Conserved hypothetical protein	10		39		3.7B
40-b	SMu1602	Conserved hypothetical protein	11		47		3.7B
40-c	SMu1602	Conserved hypothetical protein	14		54		3.7B
40-d	SMu1602	Conserved hypothetical protein	10		38		3.7B
40-e	SMu1602	Conserved hypothetical protein	15		60		3.7B
40-f	SMu1602	Conserved hypothetical protein	8		33		3.7B
54	SMu0161	Conserved hypothetical protein	3		27		3.7B
58	SMu0188	hypothetical protein	5		50		3.7B
64	SMu1315	Conserved hypothetical protein (<i>yqgA</i>)	6		12		3.7C
80	SMu0504	Conserved hypothetical protein	4		27		3.7C
82	SMu0533	Conserved hypothetical protein	8		48		3.7C
83	SMu1193	Conserved hypothetical protein	8		31		3.7C
94**	SMu1603	Hypothetical protein	5		12		3.7C, 3.7D
95	SMu1337	Conserved hypothetical protein	6		33		3.7D
102	SMu0509	Conserved hypothetical protein	3		49		3.7D
121	SMu0710	Conserved hypothetical protein	5		66		3.7A
129-a	Smu0513	Hypothetical 8.7 kDa protein	3		48		3.7A
129-b	Smu0514	Hypothetical 8.7 kDa protein	4		62		3.7A
146	SMu0307	Conserved hypothetical protein	4		25		3.7C
148	-	Hypothetical 17.4 kDa protein	5		29		3.7C
149	SMu1044	Conserved hypothetical protein	4		38		3.7C
158	SMu0112	Conserved hypothetical protein	5	6	41	45	3.7A, 3.7B, 3.8
164	SMu1887	Hypothetical protein	3		33		3.7A, 3.7B
166	SMu0901	Hypothetical 12.9 kDa protein	5		61		3.7B
171	-	Hypothetical 19.4 kDa protein	5		62		3.7D
191	SMu0334	Conserved hypothetical protein	4		56		3.7A

Table 3.1 (cont).

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function (gene)	Peptides matched ^{b)}		Coverage % ^{c)}		Figure ^{d)}
			C	EC	C	EC	
198	SMu0185	Hypothetical protein		4		24	3.8
203**	SMu0442	Conserved hypothetical protein (possible hydrolase)		4		11	3.8
204	SMu1629	Conserved hypothetical protein		6		24	3.8
212	-	Hypothetical 31.5 kDa protein		8		32	3.8
213	SMu1014	Conserved hypothetical protein		7		30	3.8
221	-	Hypothetical protein yfhK (<i>yfhK</i>)	3		24		3.7B

Table 3.2. Trypsin digested peptides from *S. mutans* LT11 proteins sequenced and identified with ESI-MS/MS

Spot ID ^{a)}	Gene ID in <i>S. mutans</i>	Putative function	Subcellular location	Sequence ^{b)}
58	SMu0188	Hypothetical protein	cellular/extracellular	SSAGG[L/I][L/I]TVTVPPE AK
197	SMu0626	Hypothetical protein	extracellular	DQG[L/I]N[L/I]AER
202-a	SMu0616	Phosphocarrier protein (histidine-containing protein)	hpr extracellular	1. FASD[L/I]T[L/I]DYK 2. PAT[L/I][L/I]VQTASK
202-b	SMu0616	Phosphocarrier protein (histidine-containing protein)	hpr extracellular	1. FASD[L/I]T[L/I]DYK 2. PAT[L/I][L/I]VQTASK
44-d	SMu1707	Mannose-specific phosphotransferase component IIAB (manL)	system cellular	[L/I]PF[L/I][L/I]QAYTER

a) Spot ID is the protein spot number on Fig 3.7 and/or 3.8.
b) The residues leucine (L) and isoleucine (I) are of the same mass and are indistinguishable with ESI-MS/MS. The underlined residue highlighted in bold is the residue matching that in the homologous protein.

Chapter 4- Metabolic proteome of *S. mutans* LT11 during acid tolerant growth.⁵

4.1 Introduction

A considerable amount of research has been devoted to studying streptococcal carbohydrate metabolism, particularly the biochemical and physiological adaptations that allow *S. mutans* to produce acids and to survive at low pH in the oral cavity. Historically, this research has employed a reductionist approach to study biochemical processes such as the allosteric regulation of enzymes and the alteration in fluxes of metabolites (Iwami and Yamada, 1980; Hamilton, 1987; Yamada, 1987; Carlsson and Hamilton, 1996; Quivey *et al.*, 2001). A number of recent reports, however, have made use of the more comprehensive strategy enabled by proteome analysis to re-evaluate the mechanism of aciduricity in *S. mutans* (Section 1.5.1). For example, an analysis by 2-DGE of ¹⁴C-labelled cellular proteins revealed 64 proteins that were up-regulated, and 49 that were down-regulated, when cells were shocked from pH 7.5 to pH 5.0. The identities of these proteins remain to be determined (Svensäter *et al.*, 2000). In a related study, 18 proteins were up-regulated and 12 down-regulated when a comparison was made between the 2-DGE proteomes of *S. mutans* grown in batch culture, without pH control, from starting pH values of 7.0 and 5.2. Of the 27 proteins identified by MS spectroscopic analysis, 13 were involved in sugar transport and central metabolism (Wilkins *et al.*, 2002). In this latter report, *S. mutans* was harvested and analysed at mid-exponential phase, at which stage the pH values of the two cultures had fallen to 6.2 and 4.7, respectively. Significantly dissimilar generation times were obtained, 1.0 h and 6.6 h, at the two pHs (Wilkins *et al.*, 2002). This variation in pH in batch culture makes an understanding of the observed changes in the proteome difficult to evaluate solely in relation to pH, since cells harvested from batch cultures have been continuously responding to an ever-changing external environment.

⁵ Some of the results presented in this chapter have already been published: Len AC, Harty DWS, Jacques NA. (2004) Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance. *Microbiology* **150**:1339-1351.

Presented in this chapter are the findings from the comparison of the proteome phenotypes and metabolic end-products of *S. mutans* grown at steady state in a chemostat at pH 7.0 or pH 5.0 in a defined artificial salivary medium. The growth pH for both pH regimes were chosen to mimic the conditions in the dental plaque of a healthy individual (pH 7.0) and that of a more susceptible host (pH 5.0). Furthermore, glucose-limited continuous-culture method was used so that cells could be sampled under strictly defined conditions at steady state, thus enabling a true comparative proteome analysis when the pH was altered.

4.2 Methods

All methods used were as described in Chapter 2 except for the following modifications which arose during ongoing refinement of techniques during the course of this study.

4.2.1 Preparation of cellular proteins

When steady state had been achieved, the bacterial contents of the culture vessel were harvested, washed and lyophilized, before aliquots (10 mg dry wt) of cells were treated with mutanolysin (Section 2.4.1). Proteins that were to be separated on acidic narrow range IPG strips (pH 4.0–6.7) were extracted as described in section 2.4.1, except that protein solubilising solution ‘MAD’ was used instead of MSS (section 3.2.2.1).

Proteins that were to be separated on basic IPG strips (pH 6–11) were obtained from the mutanolysin-treated cells (section 2.4.1) by a two-fraction solubilisation procedure. Following centrifugation of the cell lysate (12 000 g, 4 °C, 10 min), the cell pellet was stored at –20 °C, and proteins in the supernatant precipitated overnight at –20 °C with 15 % (w/v) trichloroacetic acid. Following centrifugation (12 000 g, 4 °C, 10 min) and two washes in methanol, the precipitated proteins were solubilised in 300 µl of a 1 : 1 mixture of modified solubilization solution (without ASB-14) and Cellular and Organelle Membrane Solubilising Reagent (Sigma-Aldrich), containing 1 % (v/v) Triton X-100 and 2 mM tributylphosphine. The frozen cell pellet was thawed and then resuspended by sonication (Branson Ultrasonics; 50 W, 10 X 10 s, with cooling

on ice between each burst) in 700 μ l of the same solubilization solution. Exonuclease III (150 U) was added, and the suspension incubated at room temperature (20–22 °C) for 15 min to degrade any DNA. The two cellular fractions were then combined and centrifuged at room temperature (12 000 g, 20–22 °C, 10 min). Prior to IEF, 100 μ l iodoacetamide (500 mM) was added and the mixture incubated at room temperature (20–22 °C) for 2 h.

4.2.2 2-DGE

Isoelectric focusing was carried out according to section 2.5.1. However, for basic cellular proteins, IPG strips (pH 6–11; Amersham Biosciences) were first pre-swollen with modified 2-DGE-solubilizing solution, without ASB-14 but containing 10 % (v/v) 2-propanol. Cellular proteins extracted from the equivalent of 1 mg dry cell wt were then cup-loaded onto the IPG strips.

4.3 Results and Discussion

4.3.1 Steady state growth and modified 2-DGE analysis

Substrate limited growth with constant growth rate can be achievable in a chemostat. This allows for the growth of a bacterium under a wide range of controlled steady state growth conditions in continuous culture whilst a nominated growth limiting substrate is exhausted (Pirt, 1975). Analyses showed that *S. mutans* was glucose limited at $D=0.1 \text{ h}^{-1}$, both at pH 7.0 and at pH 5.0, since 99.8 % and 99.9 %, respectively, of the glucose in the medium was utilized (Section 2.11), while all of the added amino acids remained in excess (mean utilization, 75 % at pH 7.0 and 72 % at pH 5.0)(Section 2.10).

One of the ultimate goals of comparative proteomics is to extract as many proteins as possible to maximize the proteome. To achieve this, a powerful cocktail of surfactants, chaotropes and reducing agents is needed to solubilise, disaggregate, denature and reduce the proteins (Rabilloud, 1996; Herbert, 1999; Shaw and Riederer, 2003). The addition of the reducing agent DTT and the surfactant ASB-14 increased the total number of protein spots that could be readily

discerned on narrow range pH 2-DGE gels. However, a drawback to their inclusion was selective inhibition of the extraction or subsequent separation of a small number of weakly expressed proteins that had been previously visualized and/or identified on 2-DGE gels over the pH range analysed. To enhance the extraction of basic proteins a pre-fractionation step was included prior to the protein extraction and solubilisation steps. The inclusion of this step resulted in better 2-DGE resolution of basic proteins in comparison with the method used in Chapter 3.

4.3.2 Continuous culture and 2-DGE reproducibility

The main source of error associated with using IAS quantification was the reproducibility of the 2-DGE displays themselves, since biological variation was minimized through the use of a chemostat to culture the bacteria. That the 2-DGE displays were the main source of error was confirmed by comparing the DE values of 25 randomly chosen protein spots, selected from 2-DGE displays separated on broad-range IPG strips (pH 4.0–7.0). Equivalent protein spots from triplicate samples from each of three repeat continuous cultures were analysed. The data confirmed that 2-DGE was the main source of error in determining DE values. As a consequence, triplicate experimental samples from cells grown at each pH were used for all 2-DGE analyses, and the increase in the level of expression of a protein spot was based on the change in mean DE value. A high level of reproducibility of protein spots on Sypro Ruby-stained 2-DGE gels was observed between samples obtained from the same growth pH.

4.3.3 Proteins identified by MS

A combination of steady state continuous culture technology, powerful protein solubilizing solutions and narrow range IPG strips resulted in the detection of 155 differentially expressed cellular protein spots up-regulated by more than 1.5-fold on Sypro Ruby stained 2-DGE gels following acid tolerant growth of *S. mutans* at pH 5.0. Of these, 123 were identified by MALDI-TOF analysis and 53 found to be associated with regulatory and/or stress responsive pathways (Chapter 5). The remaining 70 protein spots were associated with metabolism. These proteins

were notable for the fact that the majority were associated with glycolysis, alternative acid production and branched chain amino acid synthesis (Table 4.1)^{6,7}.

4.3.4. Glucose uptake and proton extrusion

At pH 7.0, *S. mutans* preferentially transports glucose by the Enzyme II complexes of high affinity phosphoenol-pyruvate:glucose phosphotransferase systems (PEP-PTS) capable of scavenging glucose molecules (Ellwood *et al.*, 1979). Two such systems exist in *S. mutans*, the well characterized EII^{man} and EII^{glc}, the existence of which is primarily based on physiological observations (Vadeboncoeur, 1984; Neron and Vadeboncoeur, 1987). Continuous culture studies suggest that *S. mutans* is completely devoid of EII^{man} and EII^{glc} activity at pH 5.0 (Vadeboncoeur *et al.*, 1991). At this acid pH, the bacterium preferentially utilize a non-PTS glucose permease system (Hamilton and St Martin, 1982; Dashper and Reynolds, 1990; Buckley and Hamilton, 1994), while maintaining a more alkaline cytoplasm by increasing the level of its proton translocating F₁F₀-ATPase (Hamilton and Buckley, 1991; Cvitkovitch *et al.*, 1995). The recent annotation of the *S. mutans* genome, however, does not identify a gene coding for a specific glucose permease (Ajdić *et al.*, 2002).

MALDI-TOF analysis failed to identify any intrinsic membrane proteins associated with the uptake of glucose in *S. mutans*. The failure to detect denatured intrinsic hydrophobic membrane proteins in Gram-positive bacteria is a common problem with 2-DGE proteome analysis (Chapter 3). Despite this limitation, two forms of the cytoplasmic component, EIIAB^{man} (ManL) of the EII^{man} PEP-PTS, were identified (Fig. 4.1 and Table 4.1). Unfortunately these two forms of M_r 16,060 ± 80 and 14,940 ± 30 with pI 4.44 ± 0.05 and 4.46 ± 0.02 respectively ($n = 6$), were C-terminally truncated (Fig 4.2). They represented only the EIIA portion of the protein since peptide mass fingerprint coverage was limited to the EIIA region of the protein. Although the EIIAB^{man} component of the EII^{man} PEP-PTS has been reported to be constitutively expressed in *S. mutans* (Vadeboncoeur, 1984), the truncated nature of the proteins observed in this study

⁶ Table 4.1 has been placed at the end of chapter 4 (page 119-125)

⁷ Corresponding spot numbers from triplicate gel spot images from both pH 7.0 and 5.0 regimes (Fig. 4.5, 4.6 and 4.7) have been placed at the end of chapter 4 (page 126-136)

severely limited the interpretation of the observed 6.8-fold down-regulation of these proteins at pH 5.0 and whether this down-regulation could be considered to reflect a reduction in the use of the glucose PEP-PTS under these acidic conditions (Fig. 4.1 and Table 4.1).

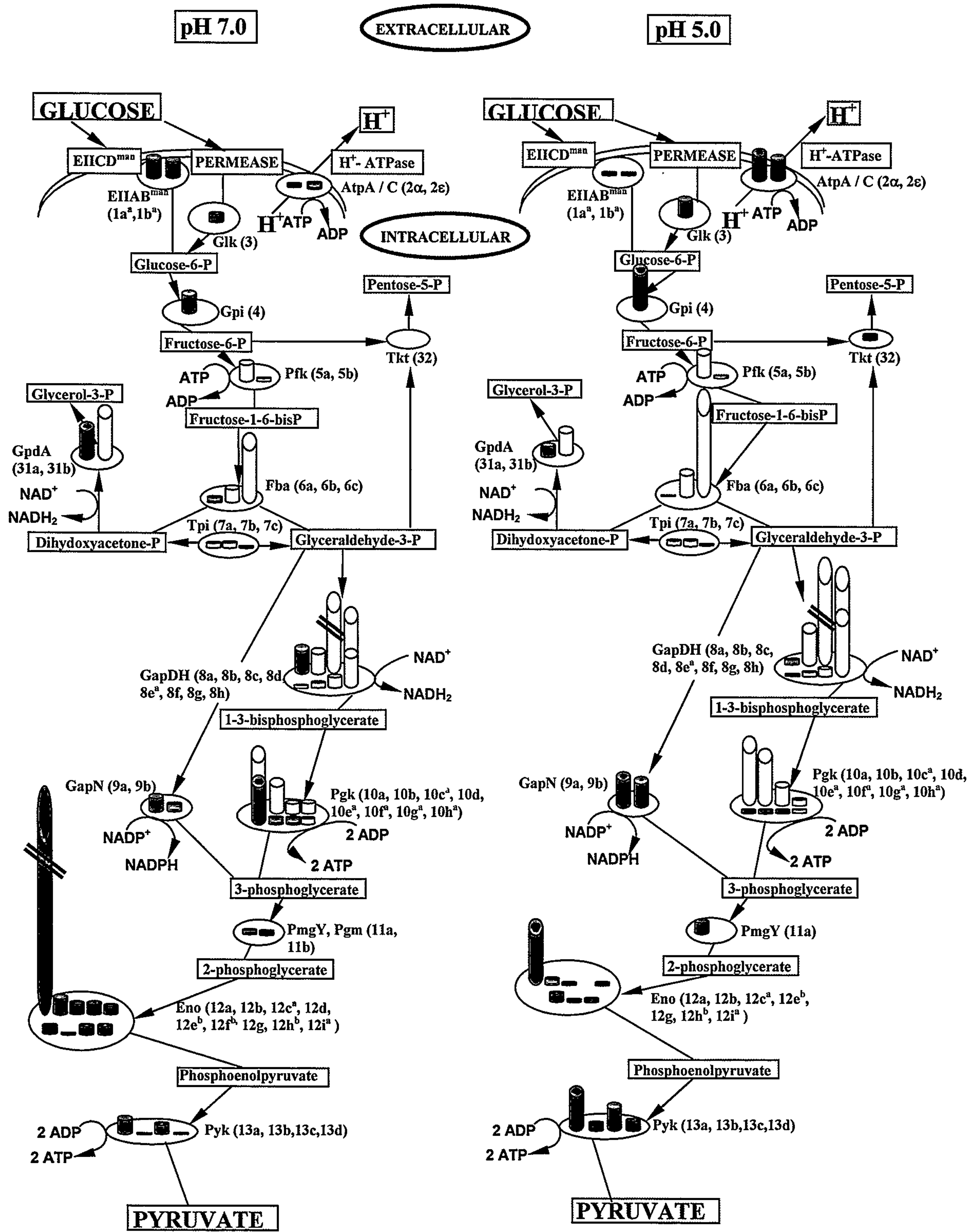
In contrast, glucokinase (Glk) required for the ATP-dependent phosphorylation of any free glucose entering the cell by way of a permease, was up-regulated by a factor of 2.1-fold at pH 5.0. However, as glucokinase has a pH optimum of approximately 8.0 (Porter *et al.*, 1982), its relative activity and stability would be affected by acidification of the cytoplasm at a growth pH of 5.0 where the value of the internal pH would be < pH 6.5 (Dashper and Reynolds, 1990; Hamilton, 1990; Iwami *et al.*, 1992). Since the optimal pH for glycolysis appears to be 7.0 (Dashper and Reynolds, 1992), the reduction in the cytoplasmic pH most likely explains why glucokinase is a rate limiting step in glycolysis when cells grown at pH 7.0 are resuspended *in vitro* at low pH (Iwami and Yamada, 1980). The observed increase in protein may therefore simply reflect a need to overcome this inhibition in cells growing at pH 5.0.

In studies of the aciduricity of *S. mutans*, the proton translocating F₁F₀-ATPase used to extrude H⁺ ions has received considerable attention since streptococcal plasma membranes are proton-permeable (Bender *et al.*, 1986). A 7.5-fold increase in the α - (AtpA) and a 3.6-fold in the ϵ - (AtpC) subunits of the F₁-component of this complex were observed at pH 5.0 (Fig. 4.1 and Table 4.1). This observation was consistent with previous data showing that the levels of the H⁺-ATPase increase in *S. mutans* in response to acidification of the environment in order to maintain a transmembrane pH gradient (Δ pH) with the interior of the cell more alkaline (Belli and Marquis, 1991; Hamilton and Buckley, 1991; Dashper and Reynolds, 1992; Quivey *et al.*, 2001).

4.3.5 Glycolysis

Glucose entering the cell by either the PEP-PTS or by the permease / glucokinase pathway enters the Embden-Meyerhof-Parnas glycolytic pathway as glucose 6-phosphate and is converted to

Figure 4.1. Comparative expression of *S. mutans* proteins involved in glucose transport and glycolysis from cells grown at pH 7.0 or at pH 5.0. The columns in the ovals represent the relative mean DE values (arbitrary units) for each protein spot identified on 2-DGE gels. DE values greater than 100,000 are shown with a height break. Protein spots were either up-regulated, (green), down-regulated (red), non-differentially expressed (< 1.5-fold difference [Grey]), or uniquely expressed (blue), relative to the alternative pH. Membrane proteins not identified by proteome analyses are shown in light blue rectangles. The numbers in parentheses correspond to the protein numbers in Table 4.1; superscripts identify protein spots as *N*-terminal (a), or *C*-terminal (b), fragments.



A

MAIGIVIASHGEFAAGIHQSGSMIFGDQEKVQVVTFMPSEGPDDLAKFN
 DAIAAFDADDEILVLADLWSGSPFNQASRVMEENSDRKFAITGLNLPML
 IQAYTERMMDAAAGVEQVAANIKEAKDGVKILPEKLQPQEEAPTTSTKK
 AAPQGAIPEGTVIGDGK LKINLARVDTRL LHGQVATNWT PASK ADR IVA
 SDTVAKDEL R KGLIKQAAPGGVKANVVPIKKLIEAAKDPRFGNTHALILF
 ETPQEALAEIEGGVPITELNIGSMAHSTGK TMVNNVISMDKDDVATFEKL
 RDLGVKFDVRKVPNDSQK DLFELIEK AHVQ

B

MAIGIVIASHGEFAAGIHQSGSMIFGDQEKVQVVTFMPSEGPDDLAKFN
 DAIAAFDADDEILVLADLWSGSPFNQASRVMEENSDRKFAITGLNLPML
 IQAYTERMMDAAAGVEQVAANIKEAKDGVKILPEKLQPQEEAPTTSTKK
 AAPQGAIPEGTVIGDGK LKINLARVDTRL LHGQVATNWT PASK ADR IVA
 SDTVAKDEL R KGLIKQAAPGGVKANVVPIKKLIEAAKDPRFGNTHALILF
 ETPQEALAEIEGGVPITELNIGSMAHSTGK TMVNNVISMDKDDVATFEKL
 RDLGVKFDVRKVPNDSQK DLFELIEK AHVQ

Figure 4.2. Peptides identified (boxed pink text) from the two forms of mannose-specific phosphotransferase system component IIAB corresponding to Fig. 4.1 and Table 4.1 spot number 1a(A) and 1b(B).

pyruvate. Each of the enzymes in this pathway varies in the number of protein spots identified on 2-DGE gels (Fig. 4.1 and Table 4.1). Previous analysis of metabolic intermediates formed in the first half of the Embden-Meyerhof-Parnas pathway when *S. mutans* is grown in continuous culture at pH 5.5 and at a $D = 0.15 \text{ h}^{-1}$ indicates that the level of glucose 6-phosphate is slightly elevated, while the levels of all other intermediates are essentially the same up to and including dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Iwami *et al.*, 1992). On this basis, the doubling of glucose-6-phosphate isomerase (Gpi) and an overall 1.5-fold increase in the level of the three isoforms of fructose 1,6-bisphosphate aldolase (Fba), similar to that previously observed in batch culture ([Wilkins *et al.*, 2002]; Fig. 4.1 and Table 4.1), may simply reflect a need to overcome the effect of cytoplasmic acidification on enzyme activity at pH 5.0. Some care should be exercised in such a simplified interpretation, however, as both fructose 6-phosphate and glyceraldehyde 3-phosphate may be required as substrates for transketolase (Tkt) in order to increase ribonucleic acid synthesis at pH 5.0 (see below).

Not only is energy in the form of ATP generated during glycolysis, but also precursors for anabolic reactions. For instance, *S. mutans* possesses a specific NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GapN) that bypasses the first ATP generating step in glycolysis in order to generate the NADPH required for reductive biosynthetic reactions ([Brown and Wittenberger, 1971; Crow and Wittenberger, 1979; Boyd *et al.*, 1995]; Fig. 4.1). This enzyme is essential, since *S. mutans* possesses neither the oxidative portion of the pentose phosphate cycle nor a transhydrogenase for the reduction of NADP by NADH (Brown and Wittenberger, 1971). A 2.0- and 3.4- fold increase in the mean amount of the two isoforms of NADP-dependent glyceraldehyde 3-phosphate dehydrogenase at pH 5.0, along with alterations in the levels of the isoforms of the NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GapDH) and phosphoglycerate kinase (Pgk) at pH 5.0 highlights the difficulty in determining the flux of carbon through the two branches of the pathway (Fig. 4.1 and Table 4.1). However, since an overall 2.7-fold increase in NADP-dependent glyceraldehyde 3-phosphate dehydrogenase at pH 5.0 represents nearly twice the increase in the cumulative levels of each of the other two enzymes in the alternative oxidative branch of glycolysis, this suggests that NADP-dependent glyceraldehyde 3-phosphate dehydrogenase may indeed be of prime importance in determining this direction. This would be in keeping with a need for a high level of anabolic activity required to maintain the structural integrity of molecules such as proteins and DNA in a non-optimal acidic cytoplasm (Crow and Wittenberger, 1979; Quivey *et al.*, 2001 [see also chapter 5]). This notion is supported by the detection of a high level of transketolase (Tkt) solely on 2-DGE gels at pH 5.0. Transketolase forms a junction in the glycolytic pathway that re-directs glyceraldehyde 3-phosphate and fructose 6-phosphate into the pentose phosphate pathway and ultimately to nucleotide synthesis (Fig. 4.1 and Table 4.1).

Three enzymes are involved in the final conversion of 3-phosphoglycerate to pyruvate. The first of these, phosphoglyceromutase, is represented by 6 alleles in the *S. mutans* genome (Ajdić *et al.*, 2002). Of the two protein products detected, one (PmgY) was up-regulated 2.7-fold at pH 5.0, while the other (Pgm) is absent at this pH (Fig. 4.1 and Table 4.1). The four isoforms of the final glycolytic enzyme, pyruvate kinase (Pyk), were also up-regulated at pH 5.0 by factors of 2.4-, 7.1-, 2.3- and 7.8- fold respectively. Pyruvate kinase is believed to be the key rate-limiting step in the regulation of glycolysis in *S. mutans* as it is activated by glucose 6-phosphate but inhibited

by inorganic phosphate (Yamada and Carlsson, 1975; Iwami and Yamada, 1980; Abbe and Yamada, 1982). However, the 93 % reduction of the dominant form of the preceding enzyme in the Embden-Meyerhof-Parnas glycolytic pathway, enolase (Eno), along with reductions in the other minor high M_r and truncated forms, would be expected to have a significant effect on carbon flow at pH 5.0, especially as the pH optimum for enolase activity is 7.5 (Bunick and Kashket, 1981).

As *S. mutans* growing in continuous culture is devoid of measurable glucose PEP-PTS activity at pH 5.0 (Vadeboncoeur *et al.*, 1991), phosphoenol-pyruvate is not required to initiate the phosphorylation cascade for this glucose uptake system. The levels of the final three enzymes in glycolysis at pH 5.0 are consistent with a reduced pool of phosphoenol-pyruvate, since the reduced levels of enolase in conjunction with an overall 2.8-fold increase in the four isoforms of pyruvate kinase would be expected to convert 2-phosphoglycerate to pyruvate without any build up of phosphoenol-pyruvate. This idea is supported by the measurement of the four intermediates, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenol-pyruvate and pyruvate in *S. mutans* grown at pH 5.5 and a $D = 0.15 \text{ h}^{-1}$, where the levels of 3-phosphoglycerate and phosphoenol-pyruvate decrease, while that of 2-phosphoglycerate remains static and the amount of pyruvate increases (Iwami *et al.*, 1992). This is further supported by another study where a fall in intracellular pH was shown to significantly increase the [pyruvate] / [phosphoenol-pyruvate] ratio (Iwami and Yamada, 1980). Phosphoenol-pyruvate is also an inhibitor of L-lactate dehydrogenase (Yamada and Carlsson, 1975; Yamada, 1987), so low levels of this metabolite would allow a higher concentration of lactate to accumulate at pH 5.0 as was indeed the case (Table 4.2).

Table 4.2. Effect of pH on yield and end-products of metabolism^{a)}

Growth	Y_{glc}		$Y_{\text{ATP}}^{\text{b)}$		IPS		End Products (mmol [mol glucose utilized] ⁻¹)				
pH	(g[dry mol ⁻¹)	wt]	(g[dry mol ⁻¹)	wt]	(μg wt)] ⁻¹	(mg[dry wt)] ⁻¹	D-Lactate	L-Lactate	Formate	Acetate	Ethanol
7.0	28.0		16.9		11.2		64	6	1156	640	368
5.0	15.1		3.9		10.9		68	760	1328	732	1688

^{a)} All data are the mean of at least three determinations.

^{b)} Determined according to the assumptions of Harty & Handley (1988).

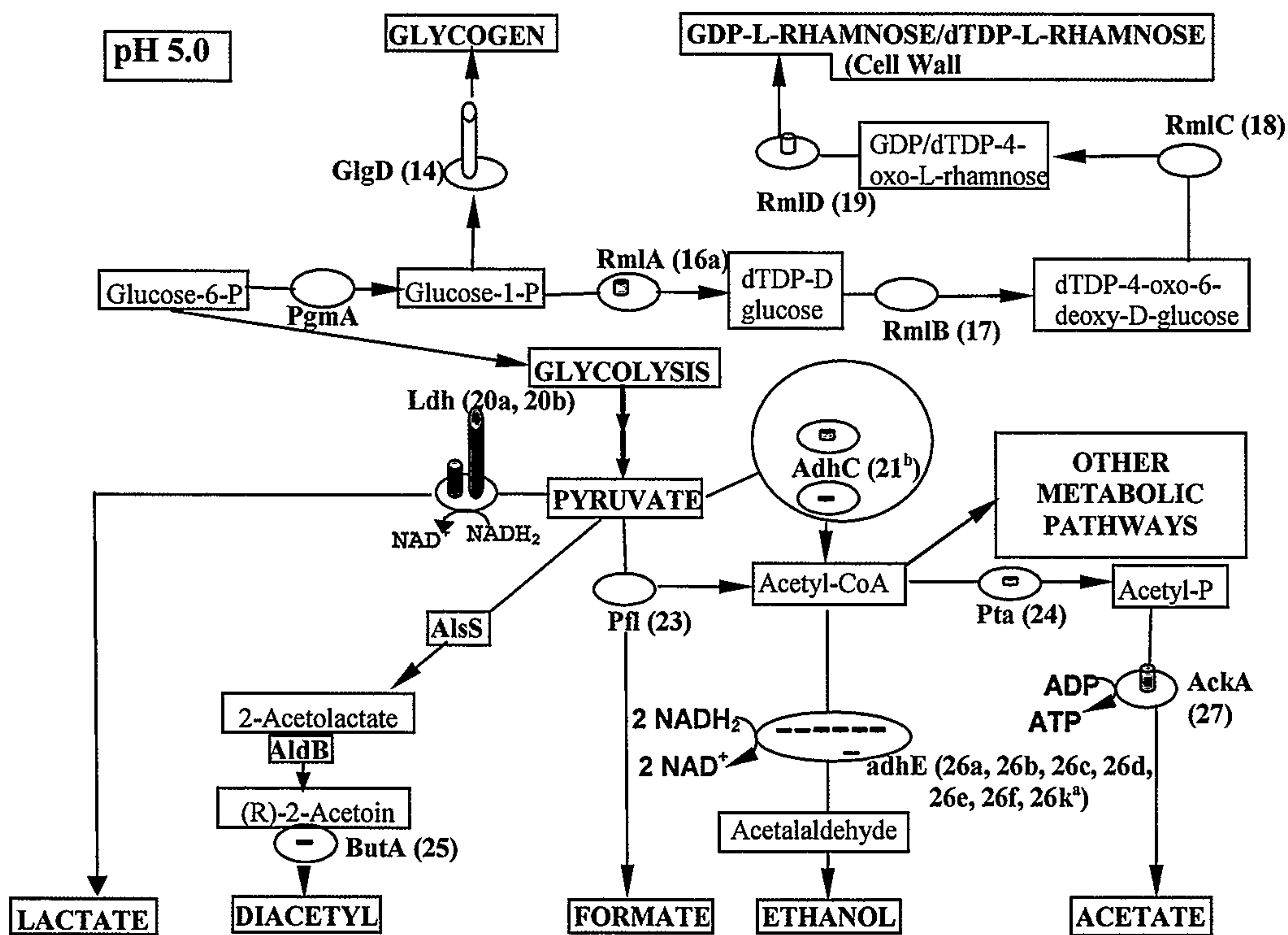
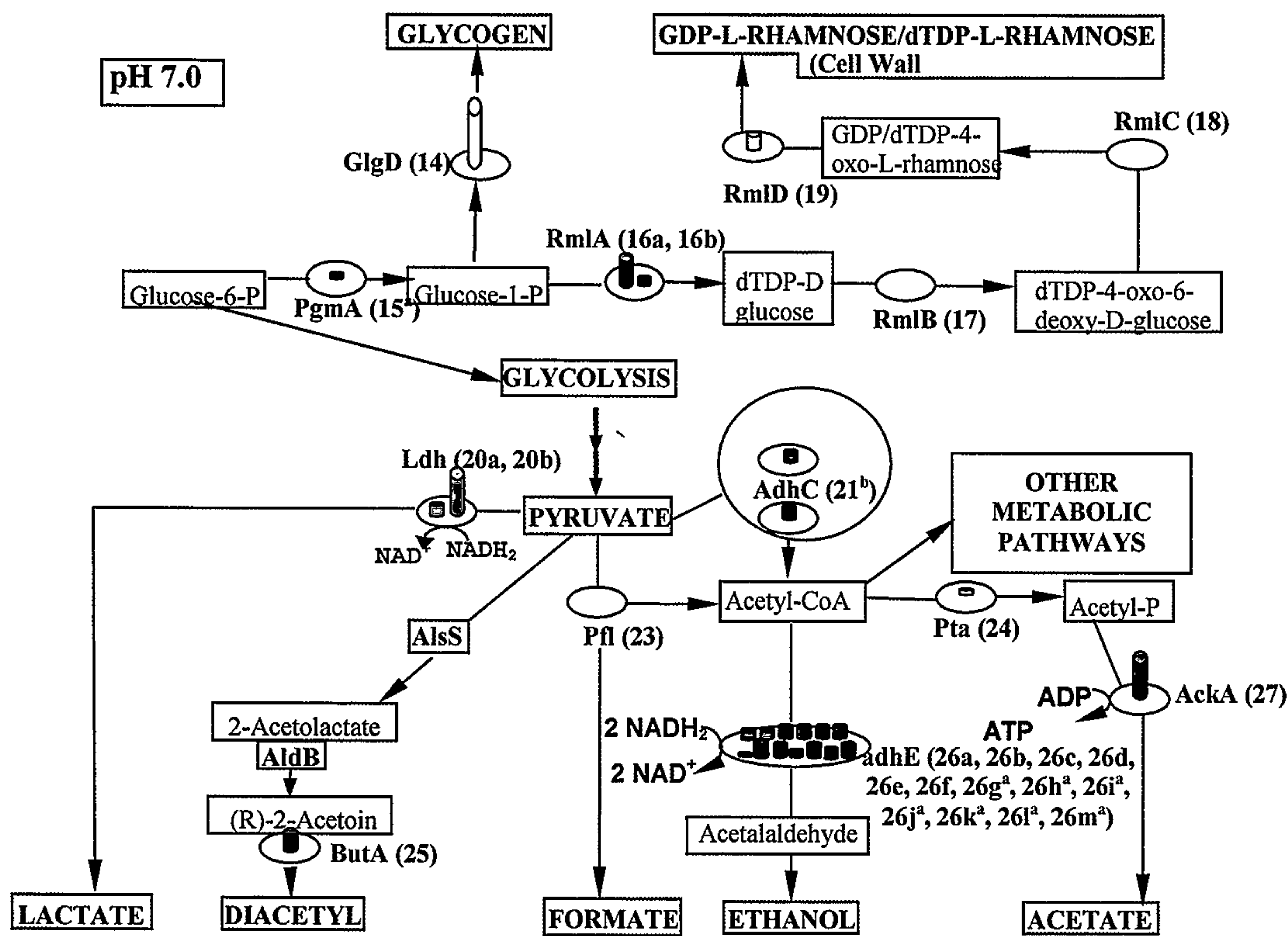
4.3.6 Regeneration of NAD

In *S. mutans*, pyruvate can be converted into a variety of acidic end products (Fig. 4.3). In an aerobic environment, *S. mutans* utilizes pyruvate dehydrogenase to convert pyruvate to acetyl-CoA (Carlsson *et al.*, 1985). Despite the fact that this enzyme should be inactive under the anaerobic conditions of growth utilized in this study, two of the three components of this multienzyme pyruvate dehydrogenase complex, the acetoin dehydrogenase E1 component, (AcoB), and a C-terminal fragment of dihydrolipoamide S-acetyltransferase (AdhC) were identified on 2-DGE gels (Fig. 4.3 and Table 4.1).

Under anaerobic conditions and in the presence of excess glucose, L-lactic acid is formed by L-lactate dehydrogenase (Ldh). By reducing pyruvate to L-lactate, NADH is oxidized to NAD and the oxidation-reduction balance of the cell is preserved. However, under anaerobic conditions when glucose is limiting, the bacterium relies on the pyruvate formate-lyase pathway which has two limbs leading to the formation of formate, acetate and ethanol (Carlsson and Griffith, 1974; Yamada and Carlsson, 1975; Yamada, 1987). The ethanol limb involves two steps in which 2NADH are oxidized to 2NAD. In *S. mutans* these two steps are accomplished by the bifunctional enzyme, alcohol-acetaldehyde dehydrogenase (AdhE), in which acetyl-CoA is first converted to acetaldehyde and then to ethanol. In the acetate limb of the pathway, an extra ATP is generated as acetyl-CoA is first converted to acetyl phosphate and then to acetate with the concomitant conversion of ADP to ATP. By appropriate use of the acetate and ethanol limbs of the pyruvate-formate lyase pathway, *S. mutans* can readily adjust NADH oxidation to actual needs. Without further anabolic diversion of the final products, the amount of formate produced by glycolysis should be equivalent to the total amount of acetate and ethanol.

Proteome analysis showed that the two isoforms of L-lactate dehydrogenase detected on 2-DGE gels were up-regulated at pH 5.0 by a factor of 2.5- and 1.7- fold compared with the levels at pH 7.0, similar to reports of a total 1.7-fold increase observed on 2-DGE gels for the three isoforms of L-lactate dehydrogenase in *S. mutans* and the five isoforms in *S. oralis* when grown without pH control in batch culture (Wilkins *et al.*, 2001; Wilkins *et al.*, 2002). The increase in L-lactate

Fig. 4.3. Comparative expression of *S. mutans* proteins involved in NADH oxidation and the alternative use of glucose 6-phosphate from cells grown at pH 7.0 or at pH 5.0. The columns in the ovals represent the relative mean DE values (arbitrary units) for each protein spot identified on 2-D gels. Protein spots were either up-regulated, (green), down-regulated, (red), non-differentially expressed (< 1.5-fold difference), (grey), or uniquely expressed, (blue), relative to the alternative pH. Proteins not identified by proteome analyses are shown in light blue rectangles, while those in tan ovals were identified in Chapter 3. The numbers in parentheses correspond to the protein numbers in Table 1; superscripts identify protein spots as *N*-terminal, (a), or *C*-terminal, (b), fragments.



dehydrogenase isoforms observed in the current study is unlikely to account for the 127-fold increase in the amount of L-lactic acid produced under acidic conditions (Table 4.2), despite the fact that L-lactate dehydrogenase has a broad optimum for catalytic activity that lies in the acidic pH range 5.0 - 6.2 similar to the pH range 6.0 - 6.5 of the cytoplasm of cells growing at pH 5.0 (Dashper and Reynolds, 1990; Hamilton, 1990; Iwami *et al.*, 1992). The more likely explanation is that the enzyme is regulated by glycolytic intermediates at the lower growth pH (Yamada and Carlsson, 1975; Takahashi *et al.*, 1982; Yamada, 1987).

While no change in the level of expression of pyruvate formate lyase was observed in the current study since the enzyme was not detected on 2-DGE gels (Fig. 4.3 and Table 4.1), the six isoforms of alcohol-acetaldehyde dehydrogenase were, on average, down regulated 7.0-fold at pH 5.0 while the numerous C-terminal truncated forms were either down regulated or missing at this pH. Acetate kinase (AckA) in the acetate limb was also down regulated by a factor of 2.1-fold. In contrast, the level of phosphotransacetylase increased 1.7-fold at pH 5.0 (Pta; Fig. 4.3 and Table 4.1). Despite the general overall reduction in protein expression observed on 2-DGE gels in these two branches of the pyruvate formate lyase pathway, increased amounts of formate, acetate and ethanol were produced at pH 5.0, with the level of ethanol being 4.6-fold greater than at pH 7.0 (Table 4.2). This observation is due mainly to the increased glycolytic rate (Hamilton IR, 1987) needed to produce ATP for proton extrusion at pH 5.0 (Bender *et al.*, 1986; Hamilton IR, 1987; Belli and Marquis, 1991; Miyagi *et al.*, 1994; Smith *et al.*, 1996). The fact that there was a futile use of carbon at pH 5.0 was reflected in the measured reduction in both ATP yield (Y_{ATP} ; Harty & Handley, 1988) from 16.9 to 3.9 g (dry wt cells)(mol ATP)⁻¹ and the cell yield (Y_{glc}) from 28.0 to 15.1 g (dry wt cells)(mol glucose)⁻¹ when the growth pH was altered from pH 7.0 to pH 5.0 (Table 4.2). While clearly not preventing the production of alternative acids, the question remains as to whether the reduction in enzyme levels in the ethanol and acetate branches of the pyruvate formate lyase pathway becomes limiting in cells growing at pH 5.0 and by so doing enhances the flow of pyruvate through the L-lactate dehydrogenase pathway.

4.3.7 Formation of branched-chain amino acids

A decrease in the formate:(acetate + ethanol) ratio from the theoretical maximum glycolytic value of 1.0 to a value of 0.56 was observed at pH 5.0 (Table 4.2). Since the concentration of acetate was similar at both pHs, the relative reduction in formate at pH 5.0 means that *S. mutans* is further metabolizing this acid, since the alternative scenario, that of an increase in ethanol production by another catabolic process, can be ruled out since ethanol can only be formed via the pyruvate-formate lyase pathway. Comparative proteome analysis suggests that the fate of some of the formate at pH 5.0 lies with the branched chain amino acid biosynthesis pathway, since an increase in the enzymes associated with this, and only this, amino acid pathway was observed on 2-DGE gels at pH 5.0 (Fig. 4.4 and Table 4.1). The connection arises as isoleucine biosynthesis requires 2-oxobutanoate, which is obtained by deamidation of serine by threonine ammonium ligase (IlvA [EC:4.3.1.19]). Serine is formed from formate by way of the one carbon donor cycle. The biosynthesis of both leucine and valine, on the other hand, relies directly on pyruvate (Fig. 4.4). Ultimately, however, branched chain amino acid biosynthesis requires both pyruvate and NADPH for the synthesis of leucine, isoleucine and valine. *De novo* synthesis of these amino acids would lessen acid production on two accounts by removing reducing equivalents both directly in the form of pyruvate and 2-oxobutanoate, and indirectly by the consumption of NADPH (Fig. 4.4). Furthermore, reducing the level of formate also reduces the $[H^+]$, since formic acid is a stronger acid (pK_a 3.75) than either lactic (pK_a 3.86) or acetic acid (pK_a 4.75).

At pH 5.0, the 4 isoforms of ketol-acid reductoisomerase (IlvC) were up-regulated 3.9-, 5.7-, 2.6- and 3.5-fold, while branched-chain amino acid aminotransferase (IlvE) and glutamine synthetase (GlnA) were up-regulated 2.7-, and 8.7-fold respectively (Fig. 4.4 and Table 4.1). The product of branched-chain amino acid aminotransferase, 2-oxoglutaric acid (α -ketoglutaric acid) is also a weaker acid than glutamate, with pK_a values of 2.8 and 5.0 compared with pK_a values of 2.2 and 4.2 for the carboxyl groups of glutamic acid. Formation of branched chain amino acids would have an added advantage as the biosynthesis of all three amino acids involves the formation of NH_3 by glutamine synthetase. This NH_3 , along with that produced from serine during the biosynthesis of isoleucine, would provide an alternative mechanism for buffering an acidic

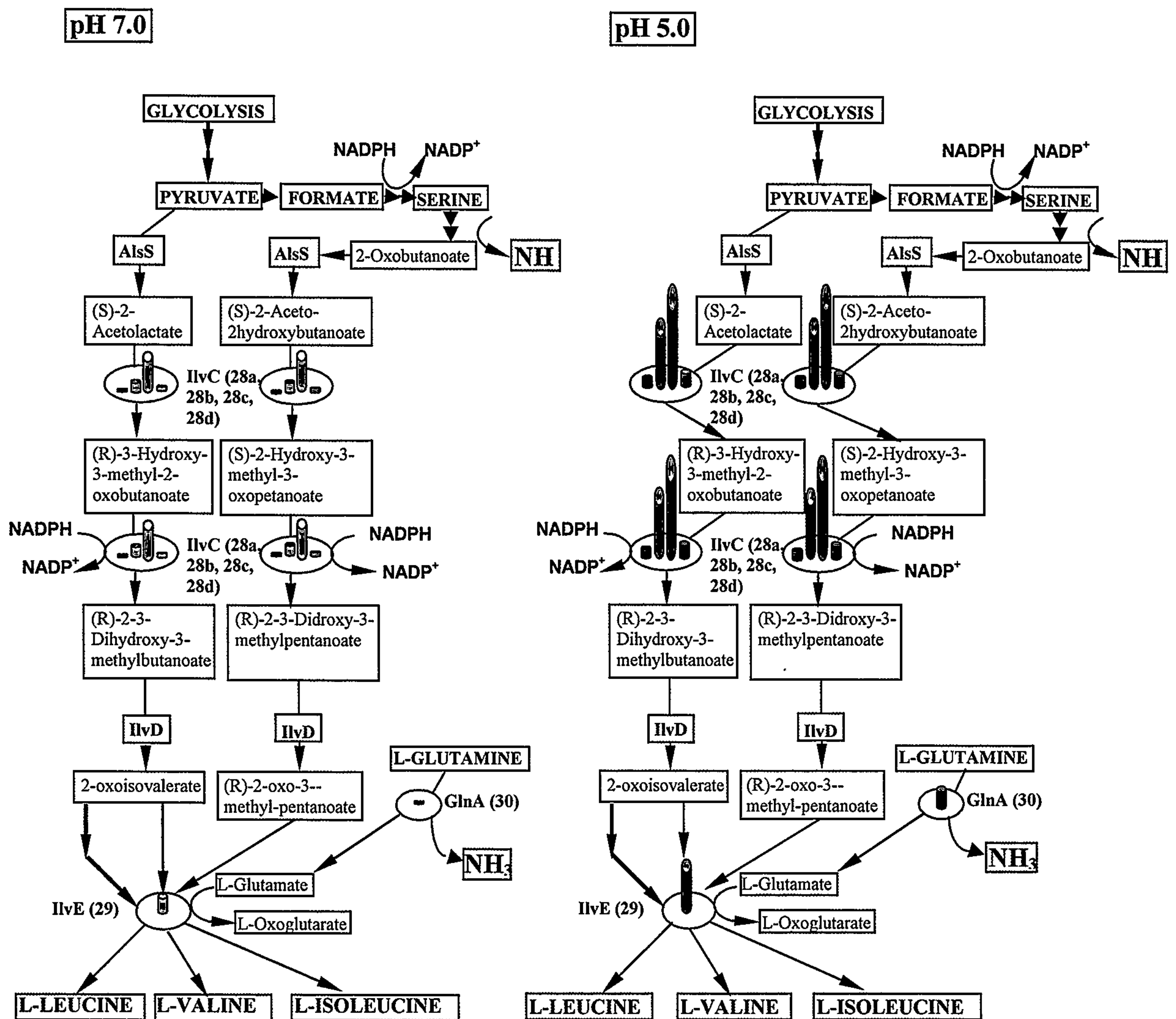


Fig. 4.4. Comparative expression of *S. mutans* proteins involved in branched chain amino acid biosynthesis from cells grown at pH 7.0 or at pH 5.0. The columns in the ovals represent the relative mean DE values (arbitrary units) for each protein spot identified on 2-D gels. Protein spots were up-regulated at pH 5.0 (green), and down-regulated at pH 7.0 (red). Proteins not identified by proteome analyses are shown in light blue rectangles. The numbers in parentheses correspond to the protein numbers in Table 4.1.

cytoplasm in *S. mutans* at a growth pH of 5.0 as the NH_3 would react with H^+ to form NH_4^+ (Fig. 4.4). Interestingly, pyruvate kinase activity has an obligatory requirement for either K^+ or NH_4^+ and a high $[\text{NH}_4^+]$ does not seem to be detrimental to the growth of oral streptococci (Abbe and Yamada, 1982; Yamada, 1987; Pitty and Jacques, 1989).

Support for a role of branched chain amino acid biosynthesis in survival of streptococci at low pH comes from two recent observations. In *Streptococcus thermophilus*, the branched chain amino acid biosynthesis pathway has been found to be necessary but not sufficient to ensure optimal growth and a role has been postulated for the pathway in maintaining internal pH (Garault *et al.*, 2000). Differential display reverse transcription-PCR has also demonstrated that branched-chain amino acid aminotransferase is up-regulated by acidic conditions in *S. mutans* and that inactivation of *ilvC* leads to retarded growth at pH 5.5 (Chia *et al.*, 2001). Further support for a role of branched chain amino acid synthesis in maintaining internal pH comes from the fact that the 2-acetolactate that is synthesized from pyruvate by acetolactate synthase (AlsS [EC:2.2.1.6]) occurs at the junction of the leucine/valine and acetoin biosynthesis pathways. The acetoin biosynthesis pathway leads to the formation of diacetyl (Fig. 4.3). While only the last enzyme in this pathway, acetoin dehydrogenase (ButA) was identified on 2-DGE gels, only 3% of the amount of the enzyme was detected at pH 5.0 (Table 4.1), suggesting that most of the 2-acetolactate would be directed into the leucine/valine biosynthesis pathway.

While the proteome data in concert with the acid and ethanol end product analyses were consistent with the formation of branched chained amino acids, the concentration of these amino acids in the continuous culture medium was only 2 % higher at pH 5.0 than at pH 7.0. Unlike other amino acid transport systems in *S. mutans*, branched chain amino acid uptake has been well studied and been shown to be driven by the proton motive force resulting in high intracellular levels (Dashper and Reynolds, 1990). Loss of these amino acids from the cell is due to passive efflux through the plasma membrane (Dashper and Reynolds, 1990). As the residual extracellular concentrations of each of the branched chain amino acids is approximately 0.3 mM at steady state in continuous culture, their cellular concentration would be as high as 10 mM (Dashper and Reynolds, 1990). Thus the failure to measure any increase in the extracellular level of these amino acids at pH 5.0 is unlikely to be a reflection on any putative increase in

cellular biosynthesis. Also worthy of note is that analysis of codon usage shows that 24.4 % of the amino acids in the proteome of *S. mutans* are branched chain amino acids, implying that there is a high biosynthetic requirement for these amino acids for protein synthesis.

4.3.8 Alterations in other biosynthetic pathways

Changes in the level of expression of individual proteins associated with incomplete metabolic pathways were also identified on 2-DGE gels. The first of these was associated with the utilization of glucose 1-phosphate following its conversion from glucose 6-phosphate by phosphoglucomutase (PgmA), a C-terminally truncated form of which was detected only at pH 7.0 (Fig. 4.3 and Table 4.1). Glucose 1-phosphate is the initial substrate for intracellular polysaccharide synthesis, little of which was synthesized at either pH consistent with glucose being limiting at $D = 0.1 \text{ h}^{-1}$ ([Hamilton IR, 1987]; Table 4.2). Glucose 1-phosphate is also the initial substrate for four enzymes associated with rhamnose biosynthesis, a sugar component of the cell wall polysaccharide of *S. mutans* (Schleifer and Kilpper-Balz, 1987). While the first of these enzymes, glucose-1-phosphate thymidyltransferase (RmlA), existed as two isoforms at pH 7.0, only one was detected at pH 5.0 and that at a 2.4-fold lower level (Fig. 4.3 and Table 4.1). The other three enzymes were not observed to be differentially expressed at the lower pH (Fig 4.3 and Table 4.1).

The second pathway was represented by only one protein, two isoforms of the NAD-dependent glycerol 3-phosphate dehydrogenase (GpdA), which forms the last step in glycerolipid degradation, directing carbon into the glycolytic pathway (Fig. 4.1). The levels of both isoforms were reduced by 2.7- and 1.9- fold at pH 5.0 (Table 4.1).

The third pathway was again represented by only one enzyme, lactoylglutathione lyase (GloA). This pathway constitutes a bypass in glycolysis that converts the reactive dicarbonyl compound, methylglyoxal, into D-lactate. Methylglyoxal is toxic as it can interact and modify both nucleic acids and proteins (Thornalley, 1996). Methylglyoxal is formed from dihydroxyacetone and glyceraldehyde-3-phosphate either with or without the enzymatic assistance of triose phosphate isomerase. While the amount of D-lactate produced by *S. mutans* was constant irrespective of

the growth pH (Table 4.2), the level of lactoylglutathione lyase was increased 2.6-fold at pH 5.0 (Table 4.1). This level of up-regulation was far less than the 51-fold increase reported for *S. mutans* grown in batch culture without pH control (Wilkins *et al.*, 2002).

4.4 Conclusion

Continuous culture in combination with enhanced 2-DGE proteome analysis using both mid-range and narrow-range IPG strips enabled the changes in expression of different isoforms of metabolic proteins to be measured during growth and tolerance of *S. mutans* in an acidic environment. The observed alterations in the levels of expression of metabolic proteins reflected the complexity required to maintain a phenotype commensurate with the survival of *S. mutans* in such an acidic environment. While this chapter has detailed the results obtained for metabolic proteins expressed under the prescribed growth conditions, the breadth of the complex acid adaptation response also includes proteins involved with other cellular events. The changes associated with other biochemical processes during acid adaptation are discussed in the next chapter (Chapter 5).

Table 4.1. Differentially expressed metabolic proteins from *S. mutans* grown at pH 7.0 or pH 5.0.

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI / M_r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
1a	SMu1707	Mannose-specific phospho-transferase system component IIAB (EC:2.7.1.69) (<i>manL</i>) ^{e)}	5.20 / 35562	4.44 ± 0.06 / 16060 ± 80	4449 ± 667	661 ± 78	4.5.4
1b	SMu1707	Mannose-specific phospho-transferase system component IIAB (EC:2.7.1.69) (<i>manL</i>) ^{e)}	5.20 / 35562	4.46 ± 0.02 / 14940 ± 30	3879 ± 589	562 ± 47	4.5.4
2a	SMu1391	ATP synthase alpha chain (EC:3.6.3.14) (<i>atpA</i>)	5.00 / 49350	4.87 ± 0.02 / 53760 ± 180	807 ± 313	6079 ± 172	4.5.2
2b	Smu1388	ATP synthase epsilon chain (EC:3.6.3.14) (<i>atpC</i>)	6.50 / 15588	6.50 / 15588 ^{d)}	1363 ± 425	4851 ± 412	4.7.2
3	SMu0493	Glucose kinase (EC:2.7.1.2) (<i>gk</i>)	4.90 / 33635	4.90 ± 0.00 / 32230 ± 200	1898 ± 172	3908 ± 367	4.5.2
4	SMu0277	Glucose-6-phosphate isomerase (EC:5.3.1.9) (<i>gpi</i>)	4.90 / 49422	4.82 ± 0.02 / 49310 ± 230	4256 ± 387	8480 ± 1443	4.5.2
5a	SMu1090	6-phosphofructokinase (EC:2.7.1.11) (<i>pfk</i>)	5.60 / 35771	5.48 ± 0.02 / 36350 ± 860	4098 ± 330	5452 ± 637	4.6.5
5b	SMu1090	6-phosphofructokinase (EC:2.7.1.11) (<i>pfk</i>)	5.60 / 35771	5.50 ± 0.00 / 36070 ± 1060	837 ± 39	639 ± 55	4.6.5
6a	SMu0088	fructose-1,6-biphosphate aldolase (EC:4.1.2.13) (<i>fbaA</i>)	5.30 / 32996	5.00 ± 0.00 / 28670 ± 140	1275 ± 81	188 ± 11	4.6.4
6b	SMu0088	fructose-1,6-biphosphate aldolase (EC:4.1.2.13) (<i>fbaA</i>)	5.30 / 32996	5.00 ± 0.00 / 29190 ± 90	3207 ± 167	5390 ± 1253	4.6.4
6c	SMu0088	fructose-1,6-biphosphate aldolase (EC:4.1.2.13) (<i>fbaA</i>)	5.30 / 32996	5.00 ± 0.00 / 29130 ± 70	12527 ± 980	20473 ± 2375	4.6.4
7a	SMu0652	Triosephosphate isomerase (EC:5.3.1.1) (<i>tpiA</i>)	5.10 / 27211	5.00 ± 0.00 / 24190 ± 80	1223 ± 218	1164 ± 360	4.6.4
7b	SMu0652	Triosephosphate isomerase (EC:5.3.1.1) (<i>tpiA</i>)	5.10 / 27211	5.00 ± 0.00 / 22160 ± 570	1295 ± 163	1395 ± 36	4.6.4
7c	SMu0652	Triosephosphate isomerase (EC:5.3.1.1) (<i>tpiA</i>)	5.10 / 27211	4.90 ± 0.00 / 21920 ± 260	493 ± 33	379 ± 74	4.6.4
8a	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	5.38 ± 0.04 / 35240 ± 310	5550 ± 73	1172 ± 138	4.6.5

Table 4.1 (cont)

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI / M_r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
8b	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	5.90 ± 0.00 / 36590 ± 350	4965 ^{e)}	7942 ^{e)}	4.7.1
8c	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	6.00 / 36068 ^{d)}	68609 ^{e)}	107697 ^{e)}	4.7.1
8d	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	5.50 ± 0.00 / 38910 ± 850	12390 ± 1097	18177 ± 6495	4.6.6
8e	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>) ^{e)}	6.00 / 36068	5.34 ± 0.03 / 28850 ± 600	690 ± 168	539 ± 85	4.6.5
8f	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	5.50 ± 0.00 / 39280 ± 760	1316 ± 320	1078 ± 211	4.6.6
8g	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	5.40 ± 0.00 / 39590 ± 540	2622 ± 433	1879 ± 181	4.6.6
8h	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12) (<i>gapDH</i>)	6.00 / 36068	6.10 ± 0.00 / 37450 ± 190	7275 ^{e)}	12491 ^{e)}	4.7.1
9a	SMu0618	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.9) (<i>gapN</i>)	5.20 / 47166	5.20 ± 0.00 / 55680 ± 380	3462 ± 65	6646 ± 145	4.6.2
9b	SMu0618	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.9) (<i>gapN</i>)	5.20 / 47166	5.20 ± 0.00 / 55390 ± 300	1672 ± 198	5756 ± 459	4.6.2
10a	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>)	5.50 / 43436	5.30 ± 0.00 / 42730 ± 460	12222 ± 500	12898 ± 1189	4.6.8
10b	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>)	5.50 / 43436	5.20 ± 0.00 / 42580 ± 320	6669 ± 835	10607 ± 1321	4.6.8
10c	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>) ^{e)}	5.50 / 43436	6.30 ± 0.00 / 26100 ± 90	2583 ± 83	4478 ± 412	4.7.2

Table 4.1 (cont)

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI/M_r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
10d	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>)	5.50 / 43436	5.25 ± 0.02 / 44570 ± 440	2616 ± 824	1511 ± 224	4.6.8
10e	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>) ^{e)}	5.50 / 43436	6.60 ± 0.00 / 29790 ± 50	3645 ± 342	262 ± 153	4.7.2
10f	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>) ^{e)}	5.50 / 43436	4.82 ± 0.02 / 38640 ± 220	1609 ± 52	798 ± 99	4.6.7
10g	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>) ^{e)}	5.50 / 43436	4.90 ± 0.00 / 38770 ± 260	1564.33 ± 64	693 ± 96	4.6.7
10h	SMu0326	Phosphoglycerate kinase (EC:2.7.2.3) (<i>pgk</i>) ^{e)}	5.50 / 43436	4.80 ± 0.00 / 38560 ± 150	1507 ± 64	799 ± 99	4.6.7
11a	SMu0543	phosphoglyceromutase (<i>pmgY</i>) (EC:5.4.2.1)	5.20 / 26050	5.02 ± 0.02 / 24650 ± 50	1039 ± 59	2758 ± 73	4.6.4
11b	SMu0067	phosphoglycerate mutase-like protein (<i>pgm</i>) (EC:5.4.2.1)	5.10 / 26526	5.10 ± 0.00 / 25390 ± 80	987 ± 108	0.0	4.6.4
12a	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>)	4.70 / 46858	4.70 / 46858 ^{d)}	166641 ± 20849	12293 ± 1423	4.5.1
12b	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>)	4.70 / 46858	4.60 ± 0.00 / 44230 ± 250	3937 ± 691	1533 ± 580	4.5.1
12c	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>) ^{e)}	4.70 / 46858	4.65 ± 0.02 / 28240 ± 160	2503 ± 104	636 ± 102	4.5.3
12d	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>)	4.70 / 46858	4.80 ± 0.00 / 42190 ± 120	2069 ± 302	0.0	4.5.1
12e	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>) ^{f)}	4.70 / 46858	4.68 ± 0.02 / 26990 ± 170	2018 ± 173	655 ± 92	4.5.3
12f	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>) ^{f)}	4.70 / 46858	4.62 ± 0.02 / 25560 ± 140	1991 ± 158	0.0	4.5.3
12g	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>)	4.70 / 46858	4.70 ± 0.00 / 41070 ± 120	541 ± 238	2006 ± 584	4.5.1
12h	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>) ^{f)}	4.70 / 46858	4.70 ± 0.00 / 27510 ± 190	2018 ± 173	655 ± 91	4.5.3
12i	SMu1140	Enolase (EC:4.2.1.11) (<i>eno</i>) ^{e)}	4.70 / 46858	4.53 ± 0.02 / 9810 ± 100	2093 ± 52	934 ± 32	4.5.4

Table 4.1 (cont)

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI / M_r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
13a	SMu1089	Pyruvate kinase (EC:2.7.1.40) (<i>pyk</i>)	5.10 / 54367	5.10 ± 0.00 / 54830 ± 150	3565 ± 849	8407 ± 2366	4.6.2
13b	SMu1089	Pyruvate kinase (EC:2.7.1.40) (<i>pyk</i>)	5.10 / 54367	5.10 ± 0.00 / 54430 ± 250	264 ± 34	1874 ± 140	4.6.2
13c	SMu1089	Pyruvate kinase (EC:2.7.1.40) (<i>pyk</i>)	5.10 / 54367	5.10 / 54367 ^{d)}	2339 ± 230	5457 ± 883	4.6.2
13d	SMu1089	Pyruvate kinase (EC:2.7.1.40) (<i>pyk</i>)	5.10 / 54367	5.05 ± 0.02 / 54410 ± 210	303 ± 60	2354 ± 1037	4.6.2
14	SMu1398	Glycogen biosynthesis protein (EC:2.7.7.27) (<i>glgD</i>)	5.70 / 42335	6.02 ± 0.02 / 43170 ± 310	9172 ^{e)}	10623 ^{e)}	4.7.1
15	SMu0985	Putative phosphoglucomutase (EC:5.4.2.8) (<i>pgmA</i>) ^{e)}	5.00 / 65152	6.13 ± 0.02 / 27100 ± 50	1044 ± 226	0.0	4.7.3
16a	SMu1330	Glucose-1-phosphate thymidyltransferase (EC:2.7.7.24) (<i>rmIA</i>)	5.30 / 32989	4.90 ± 0.00 / 28560 ± 70	4494 ± 453	1851 ± 138	4.6.4
16b	SMu1330	Glucose-1-phosphate thymidyltransferase (EC:2.7.7.24) (<i>rmIA</i>)	5.30 / 32989	5.00 ± 0.00 / 28480 ± 60	1136 ± 159	0.0	4.6.4
17	Smu1327	dTDP-glucose-4,6-dehydratase (EC:4.2.1.46) (<i>rmIB</i>)	5.80 / 39268	--- ^{g)}	--- ^{g)}	--- ^{g)}	--- ^{g)}
18	Smu1329	dTDP-4-keto-L-rhamnose reductase (EC:5.1.3.13) (<i>rmIC</i>)	4.80 / 17206	--- ^{g)}	--- ^{g)}	--- ^{g)}	--- ^{g)}
19	SMu0748	dTDP-4-keto-L-rhamnose reductase (EC:1.1.1.133) (<i>rmID</i>)	4.80 / 32398	5.00 ± 0.00 / 32040 ± 90	2429 ± 125	2396 ± 405	4.6.3
20a	SMu1018	L-lactate dehydrogenase (EC:1.1.1.27) (<i>ldh</i>)	5.10 / 35245	5.00 ± 0.00 / 35780 ± 70	1818 ± 308	4576 ± 305	4.6.3
20b	SMu1018	L-lactate dehydrogenase (EC:1.1.1.27) (<i>ldh</i>)	5.10 / 35245	5.10 ± 0.00 / 35900 ± 40	6442 ± 536	11107 ± 840	4.6.3
21	SMu0115	Dihydrolipoamide S-acetyltransferase (EC:2.3.1.12) (<i>adhC</i>) ^{f)}	5.60 / 48674	5.30 ± 0.00 / 15630 ± 180	2159 ± 430	469 ± 80	4.6.5
22	SMu1297	Acetoin dehydrogenase E1 component (EC:1.2.4.1) (<i>acoB</i>)	4.70 / 37158	4.80 ± 0.00 / 37750 ± 200	1504 ± 218	988 ± 349	4.6.7

Table 4.1 (cont)

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI / M_r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
23	SMu0363	Pyruvate formate-lyase (EC:2.3.1.54) (<i>pfl</i>)	5.20 / 87600	---	---	---	---
24	SMu0952	phosphotransacetylase (EC:2.3.1.8) (<i>pta</i>)	4.80 / 36369	4.90 ± 0.00 / 31230 ± 130	578 ± 72	1000 ± 36	4.6.7
25	SMu1207	Putative acetoin dehydrogenase (EC:1.1.1.5) (<i>butA</i>)	6.00 / 27524	6.14 ± 0.02 / 28010 ± 260	2910 ± 87	91 ± 91	4.7.3
26a	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>)	5.60 / 96993	5.40 ± 0.00 / 89870 ± 1110	1660 ± 370	285 ± 60	4.6.1
26b	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>)	5.60 / 96993	5.42 ± 0.18 / 89590 ± 1020	2165 ± 260	286 ± 36	4.6.1
26c	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>)	5.60 / 96993	5.48 ± 0.017 / 89400 ± 1380	2636 ± 337	365 ± 257	4.6.1
26d	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>)	5.60 / 96993	5.50 ± 0.00 / 89520 ± 1320	2787 ± 502	1340 ± 35	4.6.1
26e	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>)	5.60 / 96993	5.52 ± 0.02 / 88900 ± 1430	4271 ± 563	1816 ± 236	4.6.1
26f	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>)	5.60 / 96993	5.60 ± 0.00 / 89000 ± 1780	3678 ± 125	1321 ± 429	4.6.1
26g	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	5.40 ± 0.00 / 48650 ± 120	756 ± 231	0.0	4.6.6
26h	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	6.00 ± 0.00 / 63870 ± 1080	3506 ± 898	0.0	4.7.1
26i	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	6.10 ± 0.00 / 65090 ± 660	2797 ± 186	0.0	4.7.1

Table 4.1 (cont)

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI / M _r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
26j	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	6.10 ± 0.00 / 57190 ± 650	1441 ± 133	0.0	4.7.1
26k	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	6.10 ± 0.00 / 55660 ± 650	2898 ± 206	53 ± 53	4.7.1
26l	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	6.00 ± 0.00 / 60440 ± 220	2325 ± 293	0.0	4.7.1
26m	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC:1.2.1.10) (<i>adhE</i>) ^{e)}	5.60 / 96993	6.10 ± 0.00 / 40860 ± 290	2906 ± 340	0.0	4.7.1
27	Smu1186	Acetate Kinase (EC:2.7.2.1) (<i>ackA</i>)	6.10 / 43732	6.10 ± 0.00 / 43650 ± 480	8651 ± 477	4084 ± 381	4.7.1
28a	SMu0211	Ketol-acid reductoisomerase (EC:1.1.1.86) (<i>ilvC</i>)	5.10 / 37285	5.00 ± 0.00 / 37260 ± 90	461 ± 97	1818 ± 195	4.6.3
28b	SMu0211	Ketol-acid reductoisomerase (EC:1.1.1.86) (<i>ilvC</i>)	5.10 / 37285	5.05 ± 0.02 / 37210 ± 90	1955 ± 372	11075 ± 1737	4.6.3
28c	SMu0211	Ketol-acid reductoisomerase (EC:1.1.1.86) (<i>ilvC</i>)	5.10 / 37285	5.10 / 37285 ^{d)}	6250 ± 389	16212 ± 797	4.6.3
28d	SMu0211	Ketol-acid reductoisomerase (EC:1.1.1.86) (<i>ilvC</i>)	5.10 / 37285	5.15 ± 0.02 / 37820 ± 60	772 ± 16	2731 ± 644	4.6.3
29	SMu1099	branched-chain amino acid aminotransferase (EC:2.6.1.42) (<i>ilvE</i>)	5.10 / 37732	5.10 ± 0.00 / 40180 ± 130	3133 ± 223	8388 ± 536	4.6.3
30	SMu0329	Glutamine synthetase, type I (EC:6.3.1.2) (<i>glnA</i>)	5.20 / 49955	5.20 ± 0.00 / 47700 ± 250	401 ± 139	3481 ± 146	4.6.2
31a	SMu0290	Glycerol-3-phosphate dehydrogenase (EC:1.1.1.8) (<i>gpdA</i>)	6.00 / 37826	6.06 ± 0.02 / 35430 ± 180	6971 ± 455	2588 ± 356	4.7.3
31b	SMu0290	Glycerol-3-phosphate dehydrogenase (EC:1.1.1.8) (<i>gpdA</i>)	6.00 / 37826	6.00 ± 0.0 / 33690 ± 80	10202 ± 1832	5385 ± 992	4.7.3

Table 4.1 (cont)

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI / M _r)		DE Value ^{a)}		Figure ^{b)}
			Theoretical	Observed ^{a)}	pH 7.0	pH 5.0	
32	SMu0262	Probable transketolase (EC:2.2.1.1) (<i>tkt</i>)	5.00 / 71076	5.00 ± 0.00 / 72910 ± 490	0.0	1145 ± 108	4.6.2
33	SMu1458	Lactoylglutathione lyase (EC:4.4.1.5) (<i>gloA</i>)	4.90 / 15271	5.07 ± 0.02 / 17980 ± 90	991 ± 111	2599 ± 86	4.6.4

^{a)} Mean ± SEM (*n* = 3).

^{b)} Figure containing the corresponding protein spot.

^{c)} N-terminus of protein.

^{d)} Part of the complement of random landmark proteins used to align gels and which were used for the calculation of the observed pI and M_r in accordance with the requirement of the z3 software.

^{e)} DE value obtained from a single 2-DGE gel. All other proteins were identified on triplicate gels.

^{f)} C-terminus of protein.

^{g)} Previously detected by 2-DGE proteome analysis (Chapter 3).

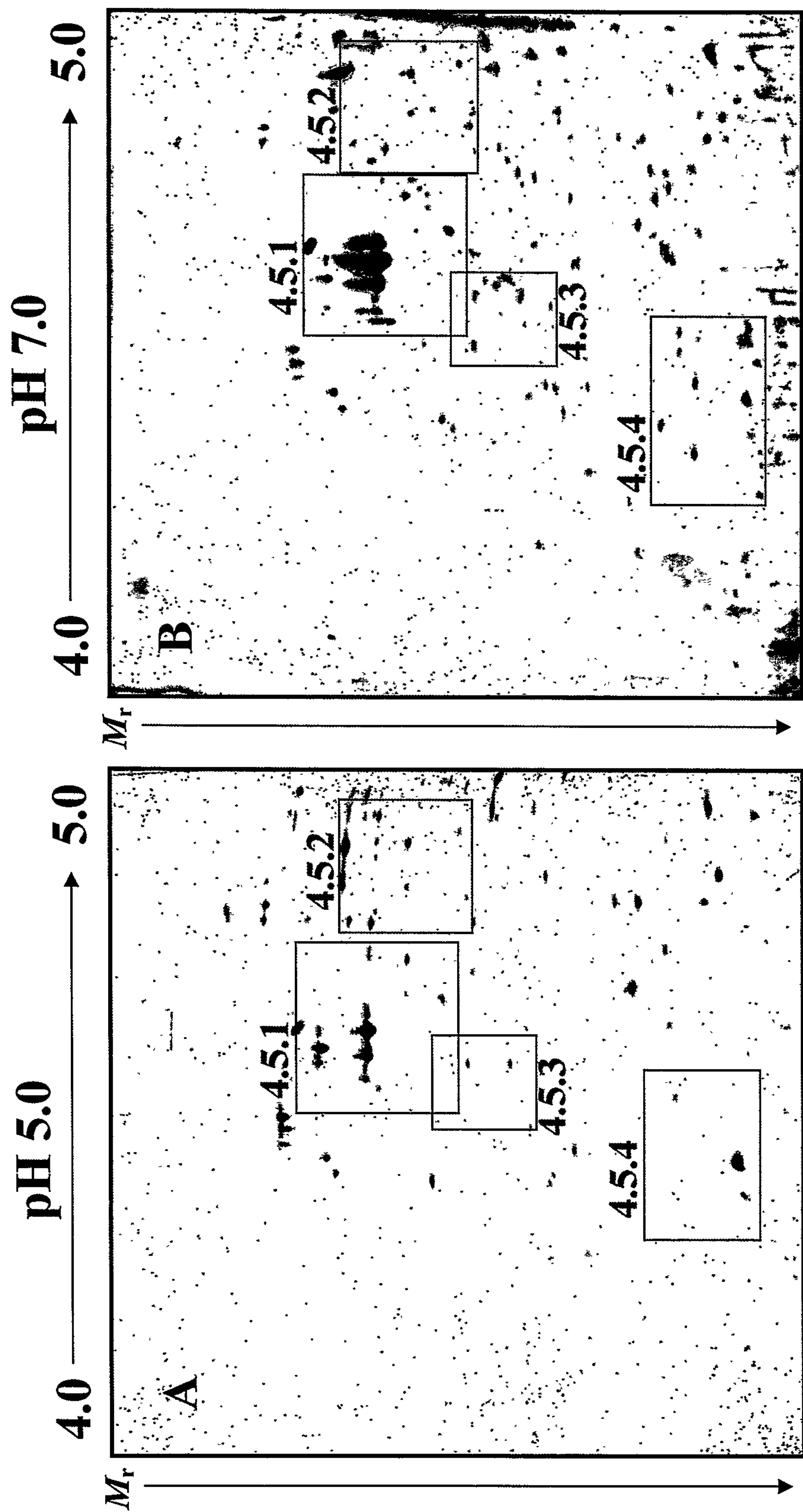
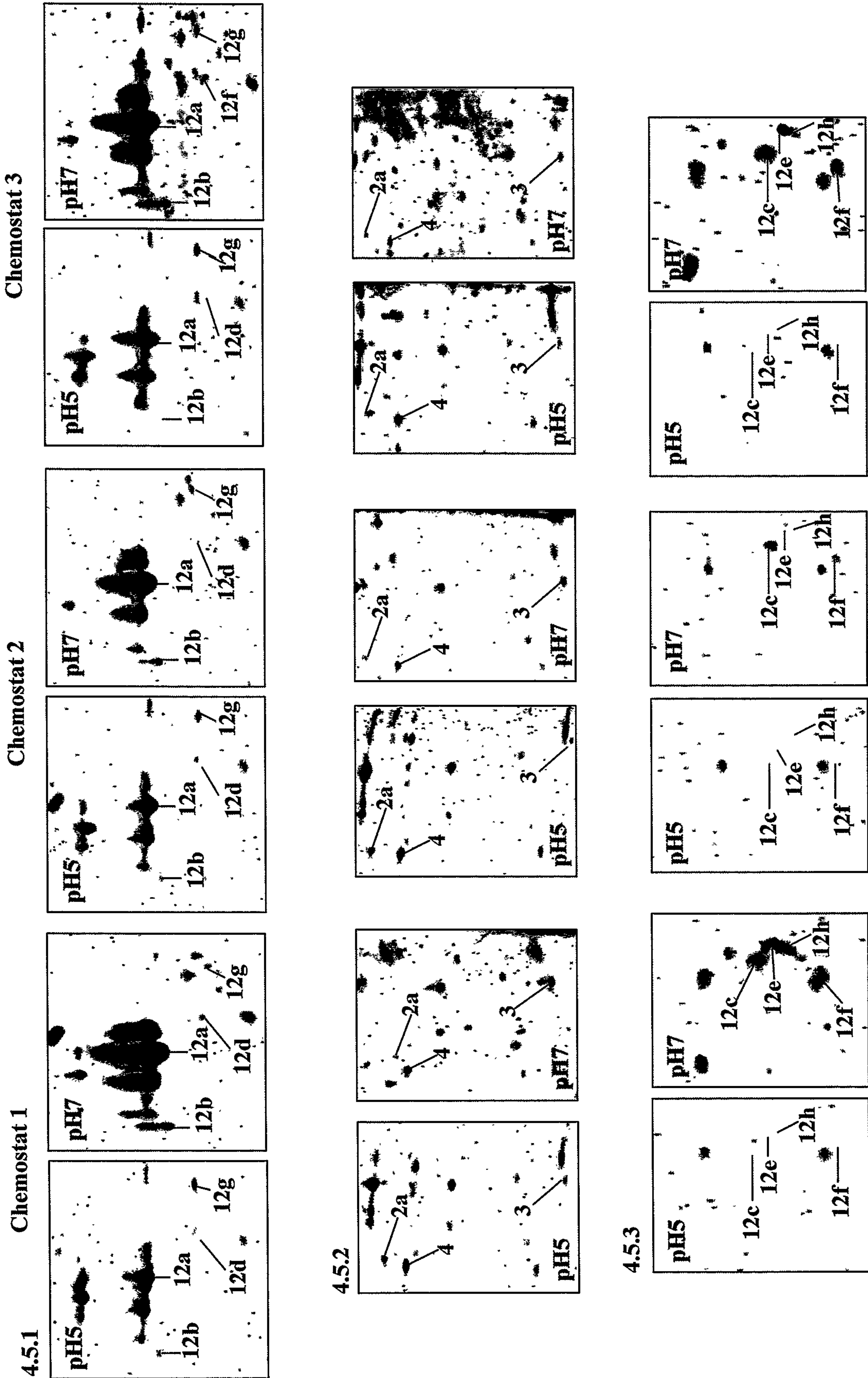


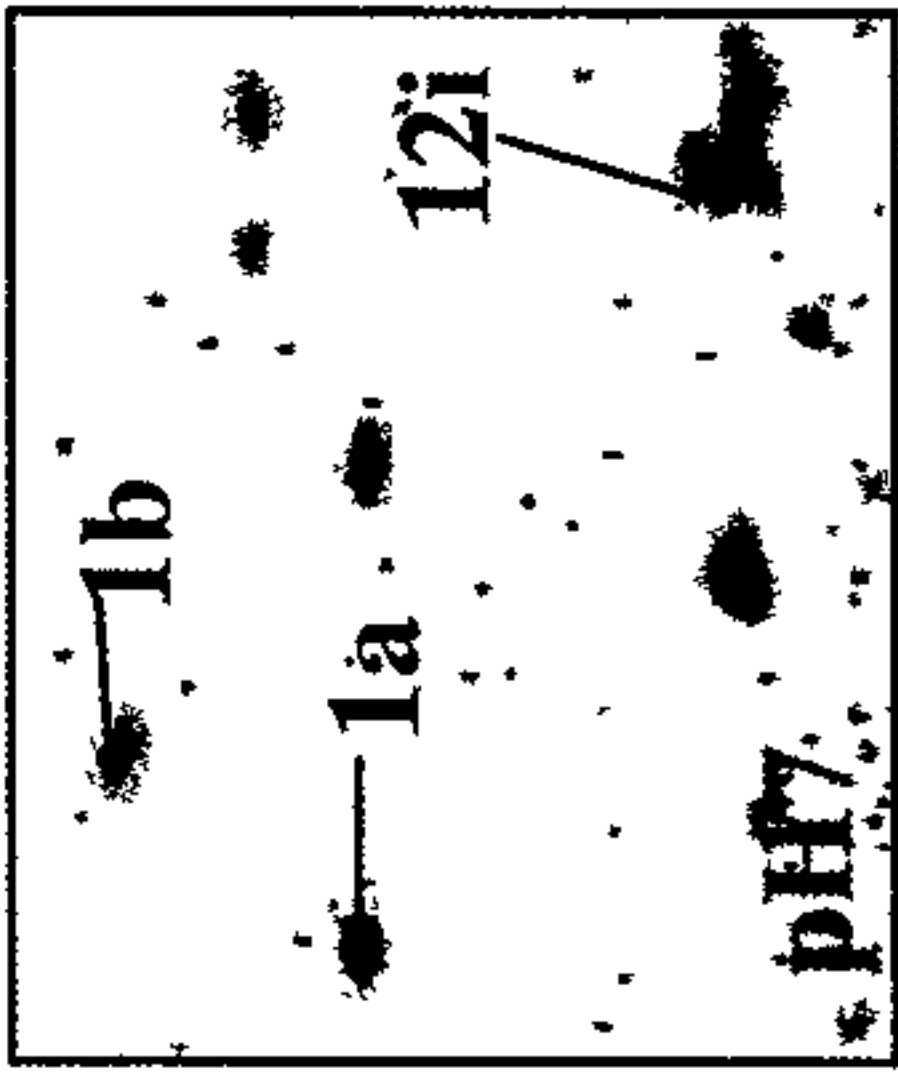
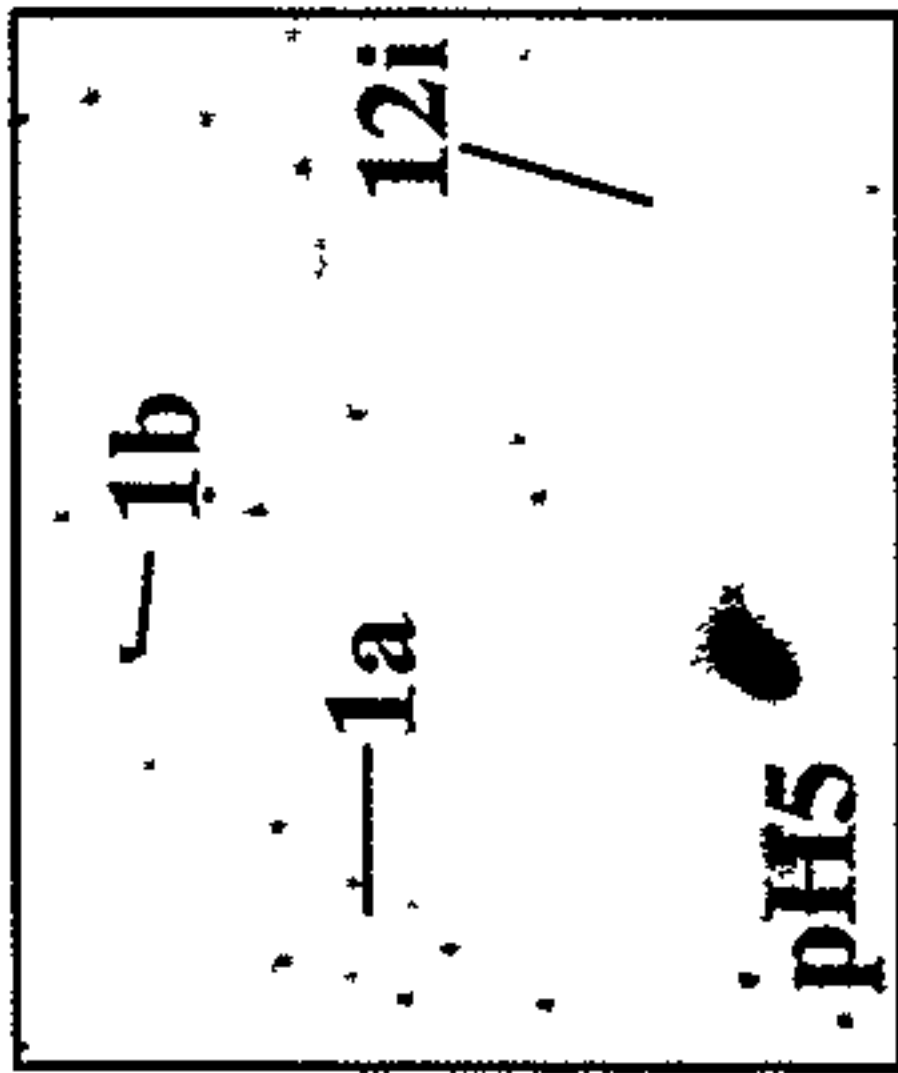
Figure 4.5. 2-DGE Sub-proteome maps (pH 4.0 – 5.0) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas (4.5.1, 4.5.2, 4.5.3 and 4.5.4) depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed areas, corresponding to their respective chemostat 2-DGE gels, are illustrated in 4.5C with spot numbers corresponding to those in Table 4.1.

4.5 C

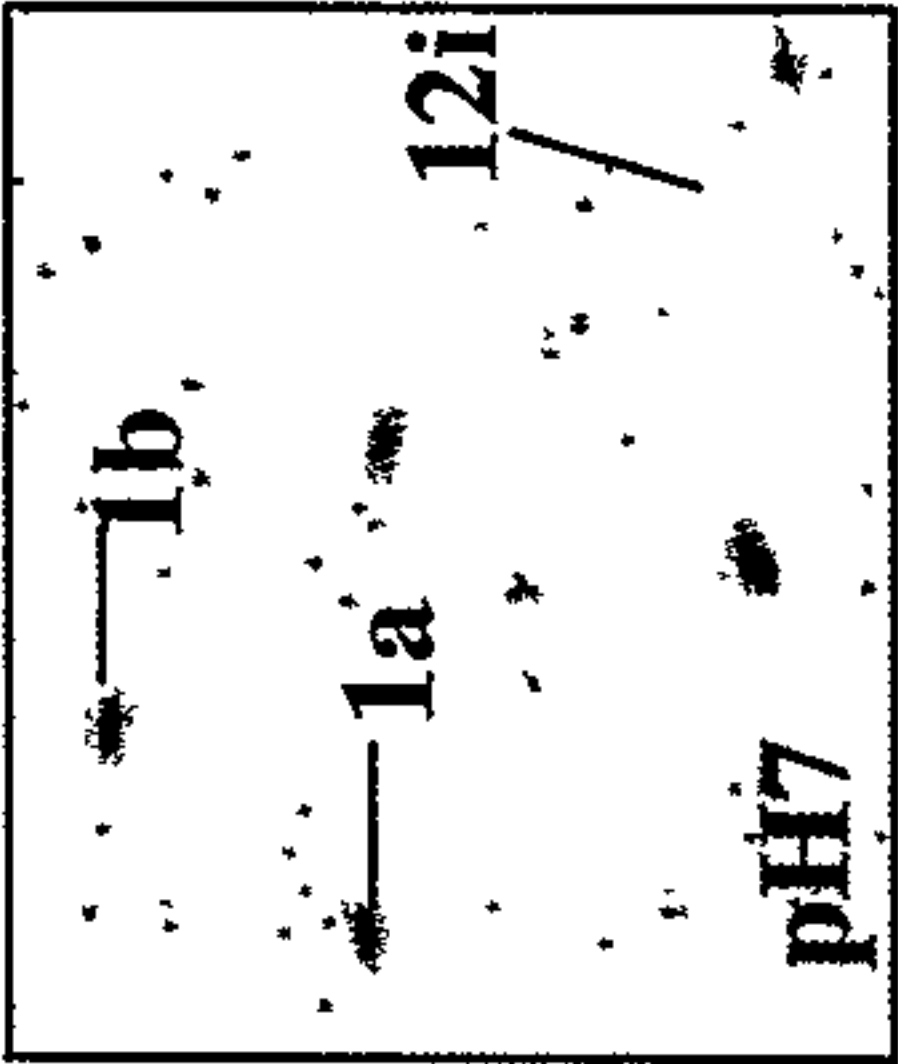
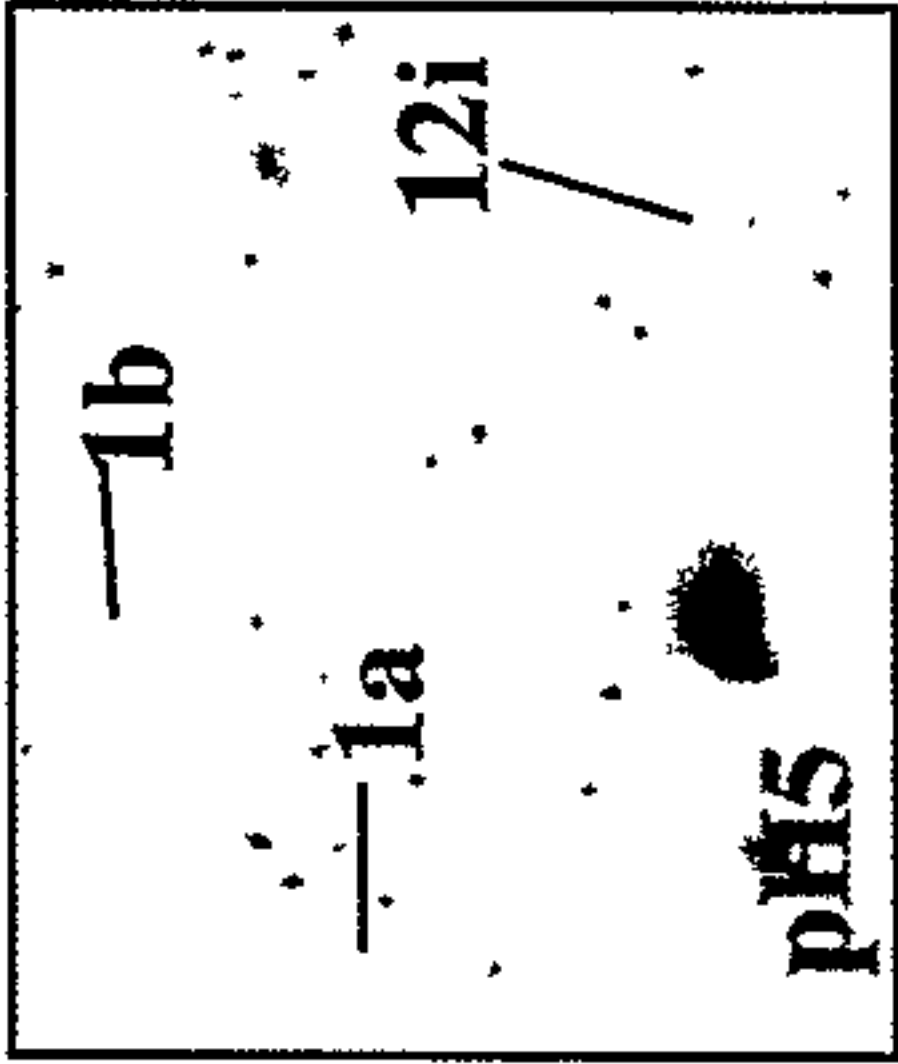


4.5 C (cont)

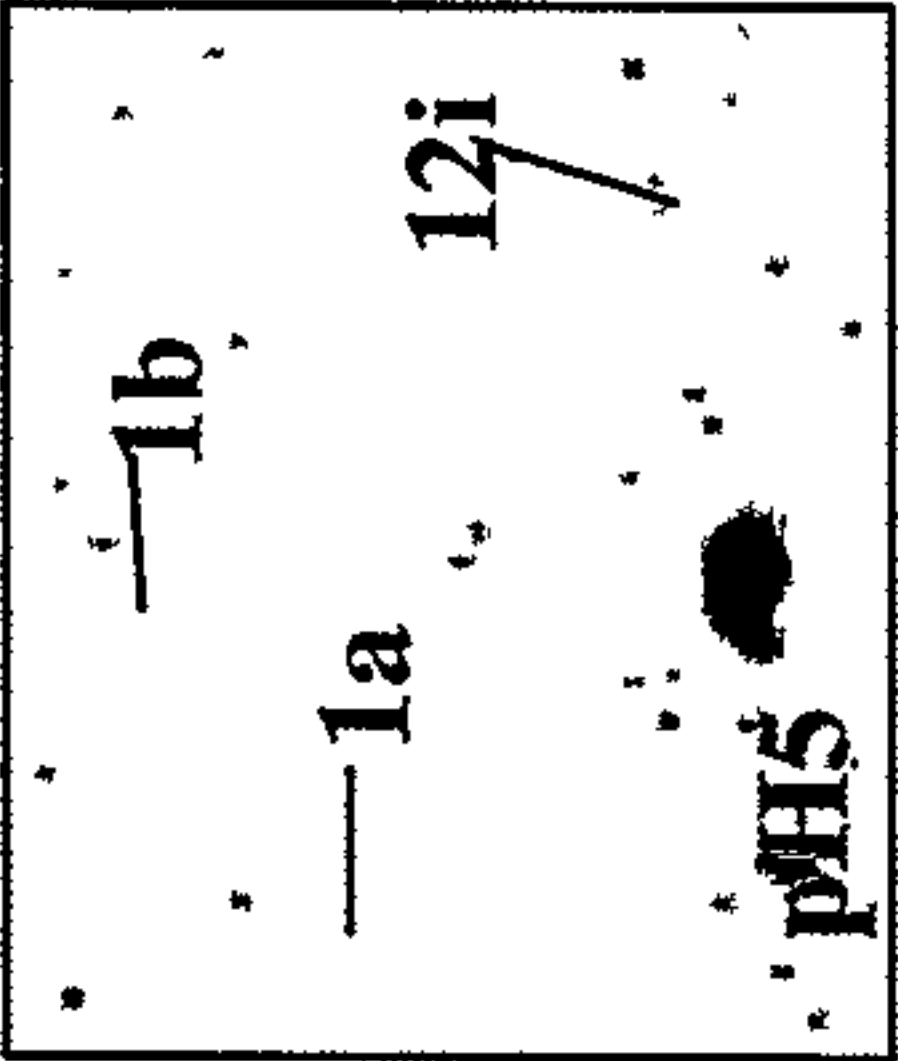
4.5.4 Chemostat 1



Chemostat 2



Chemostat 3



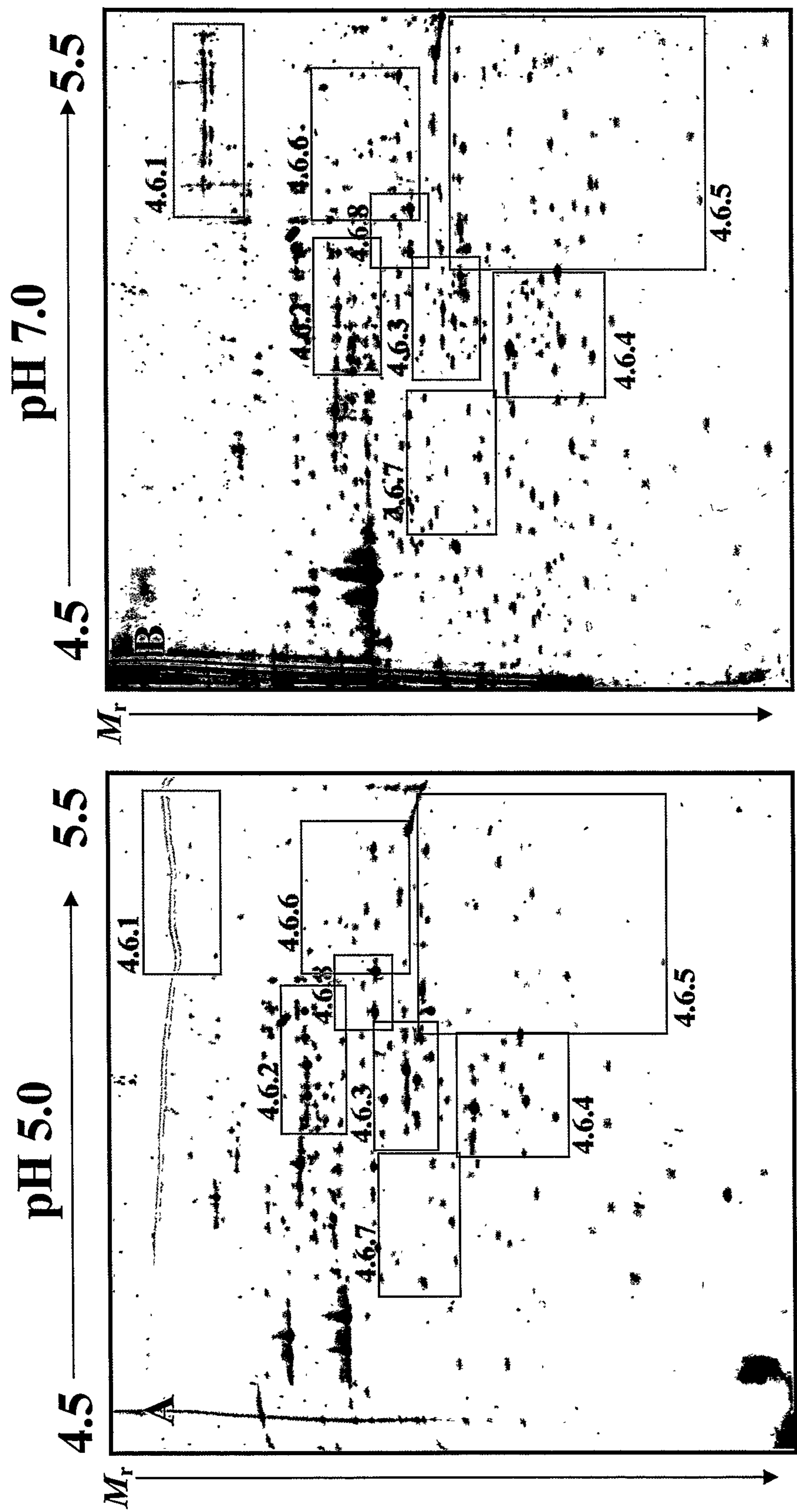
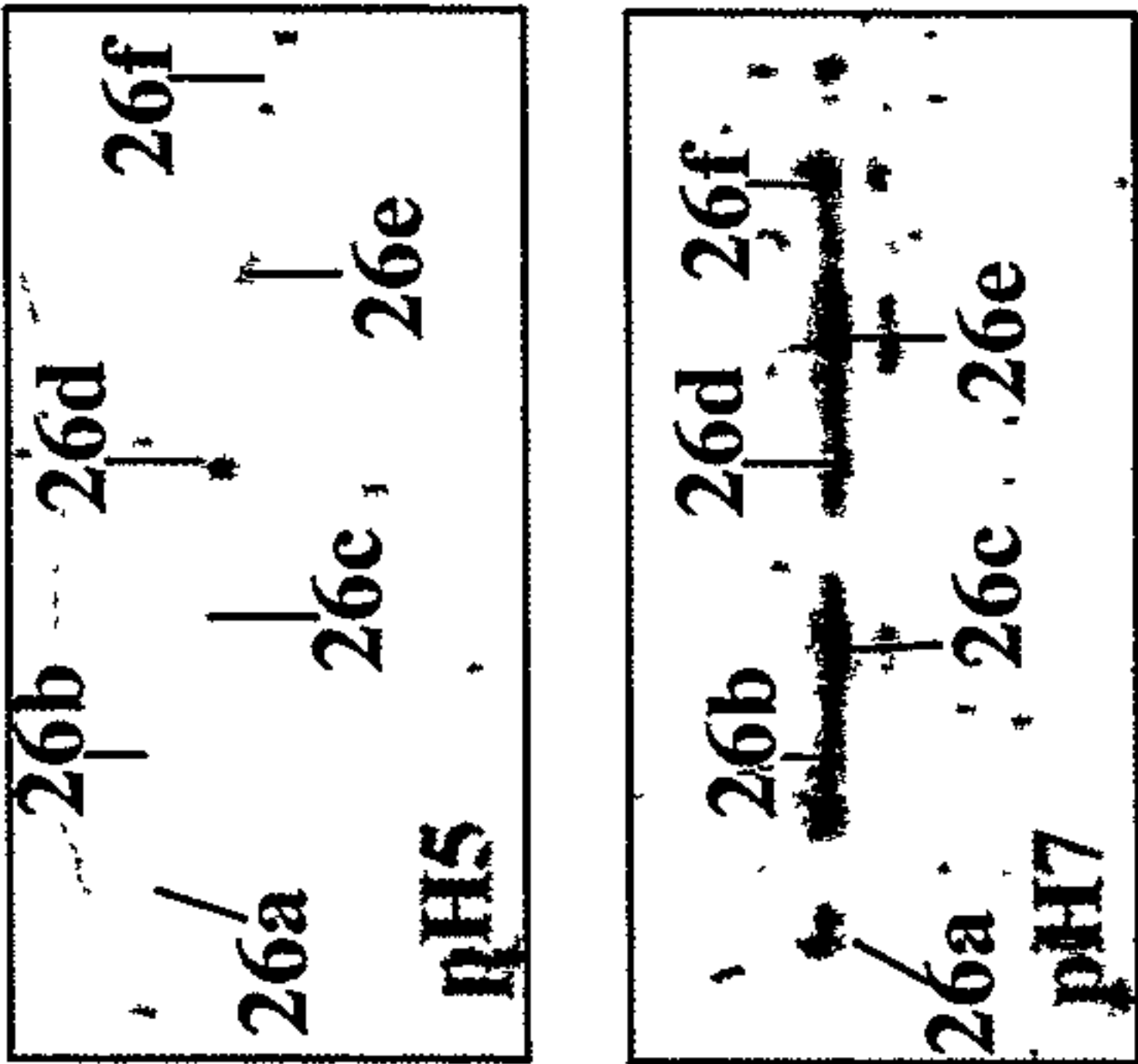


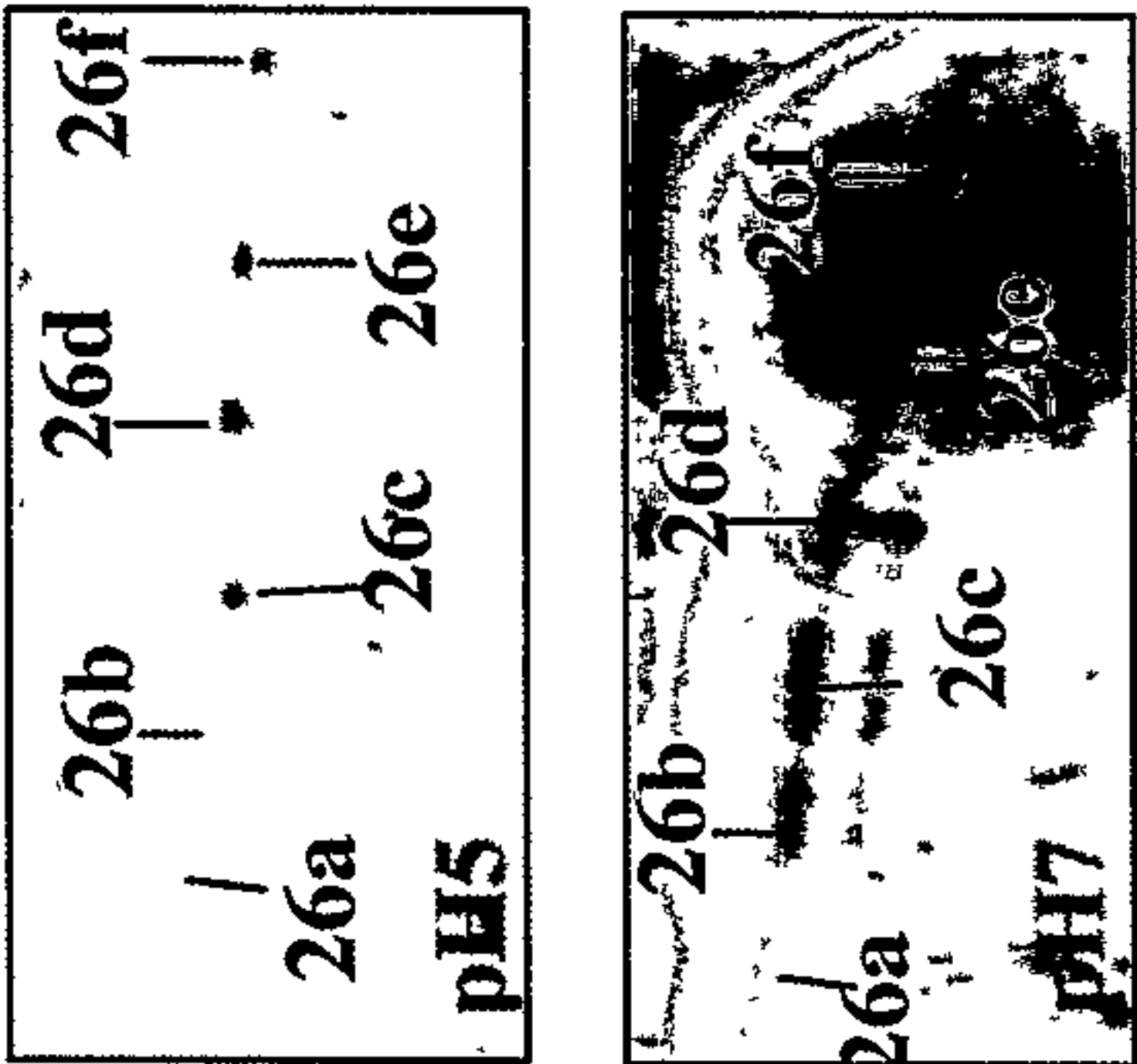
Figure 4.6. 2-DGE Sub-proteome maps (pH 4.5 – 5.5) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas (4.6.1, 4.6.2, 4.6.3, 4.6.4, 4.6.5, 4.6.6, 4.6.7, and 4.6.8) depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed areas, corresponding to their respective chemostat 2-DGE gels, are illustrated in 4.6C with spot numbers corresponding to those in Table 4.1.

4.6C

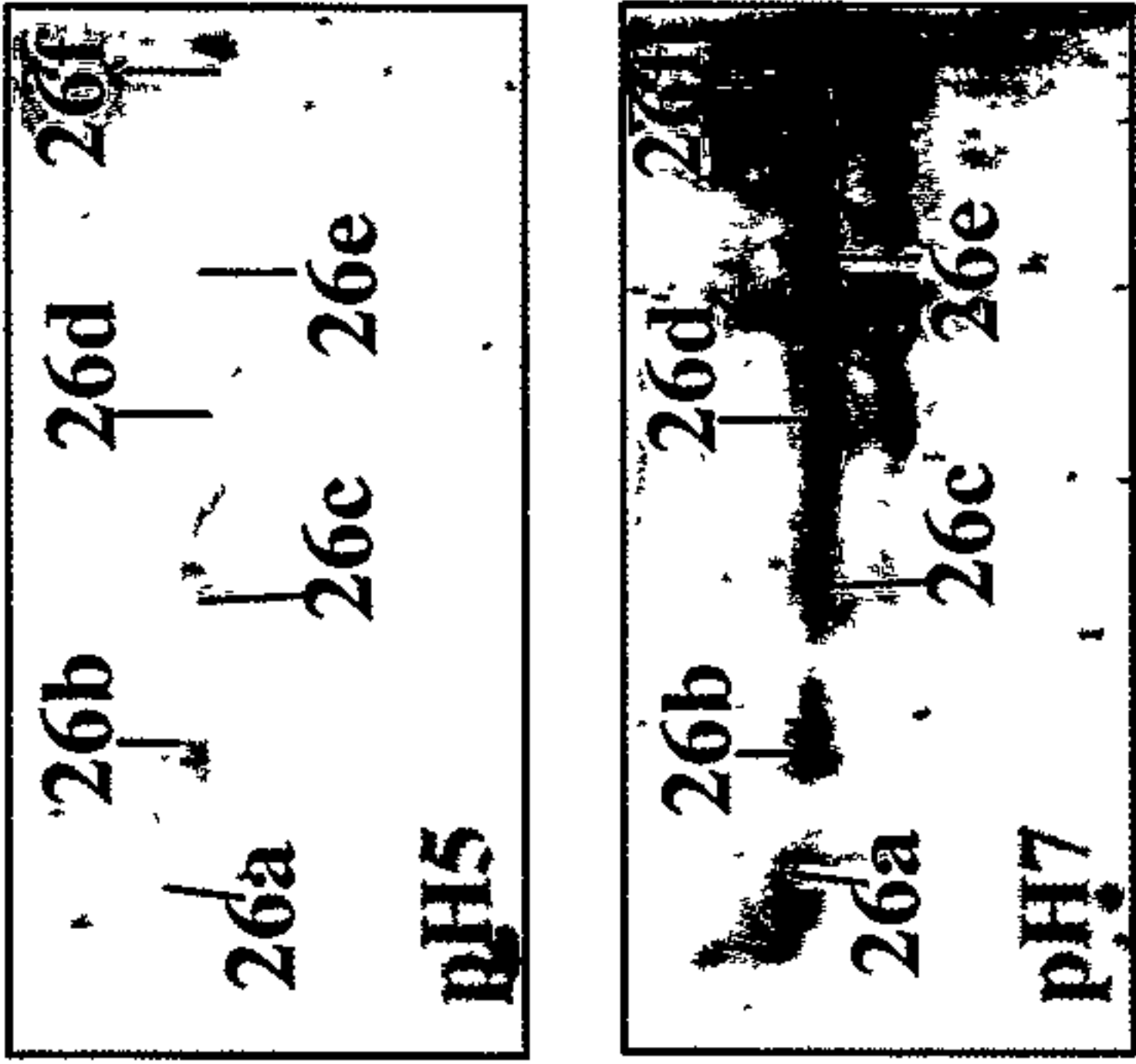
4.6.1 Chemostat 1



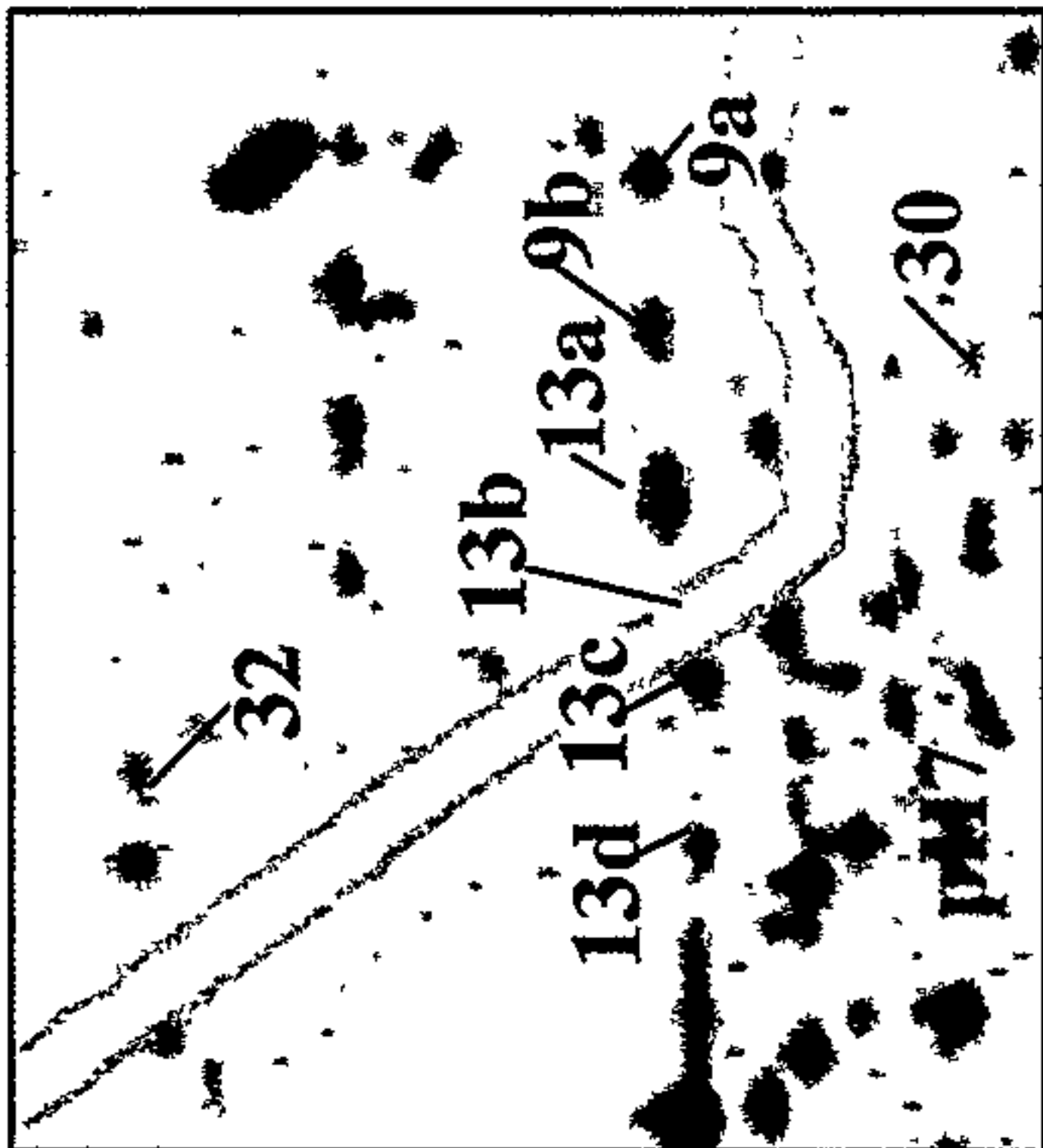
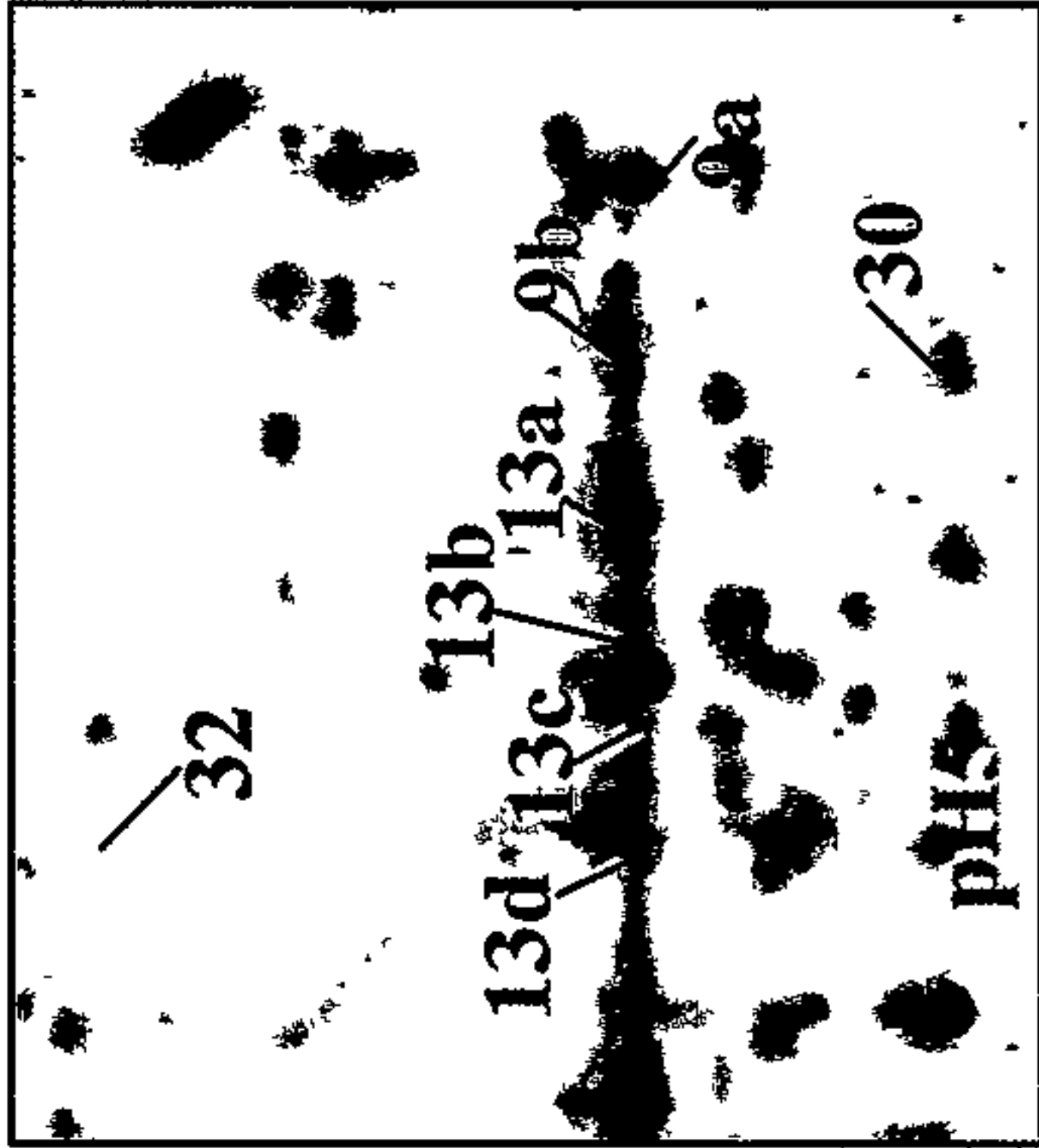
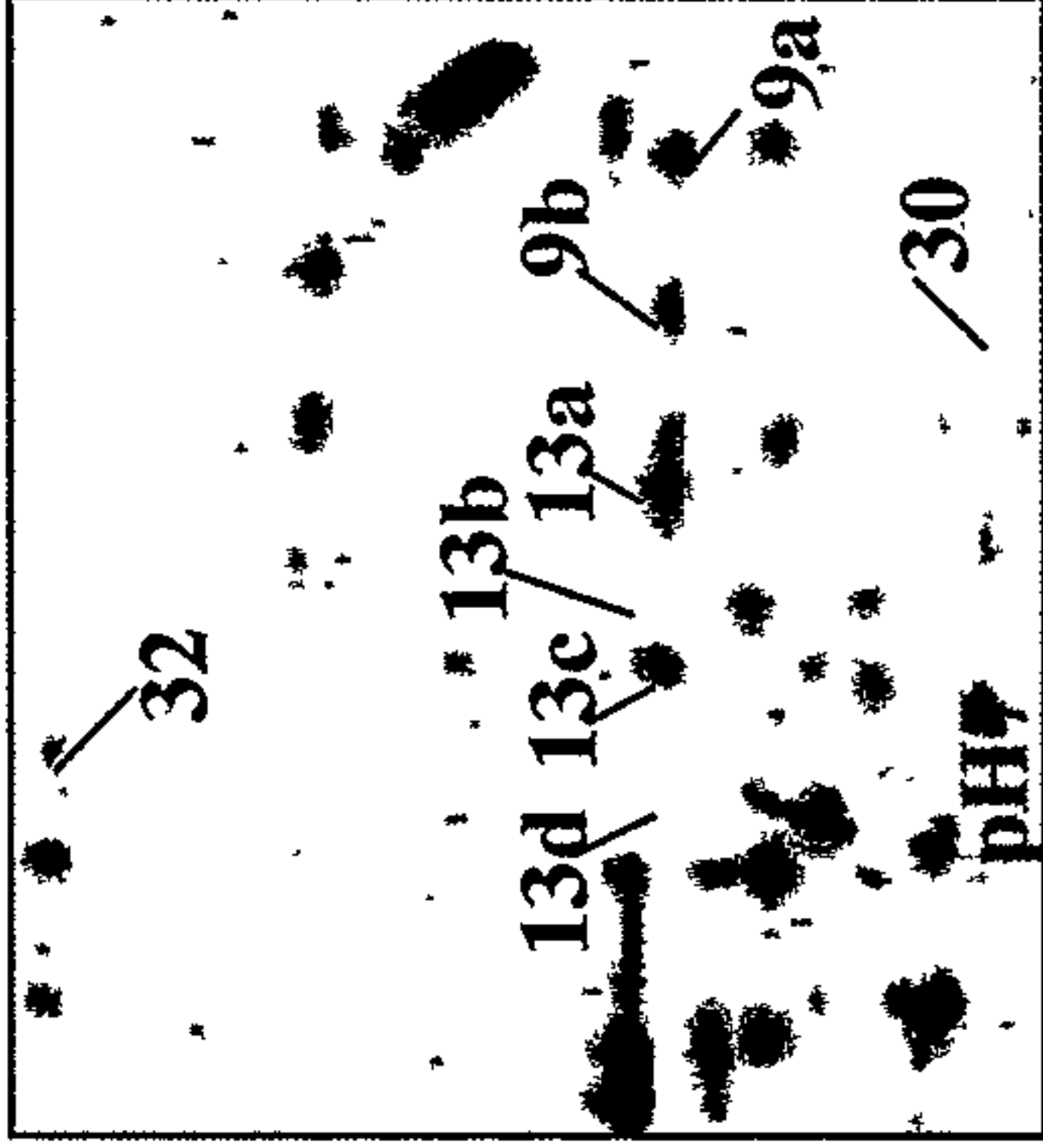
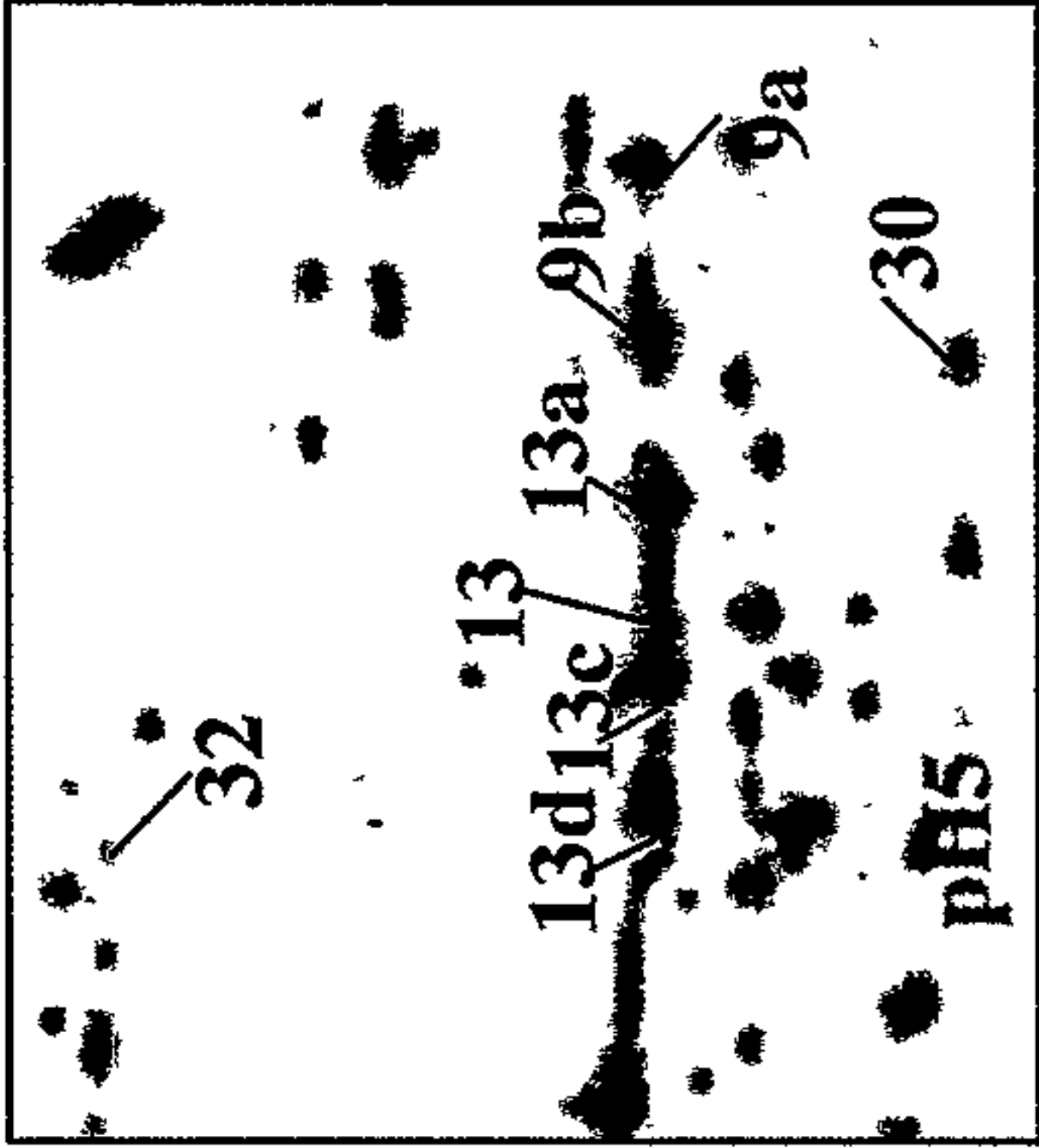
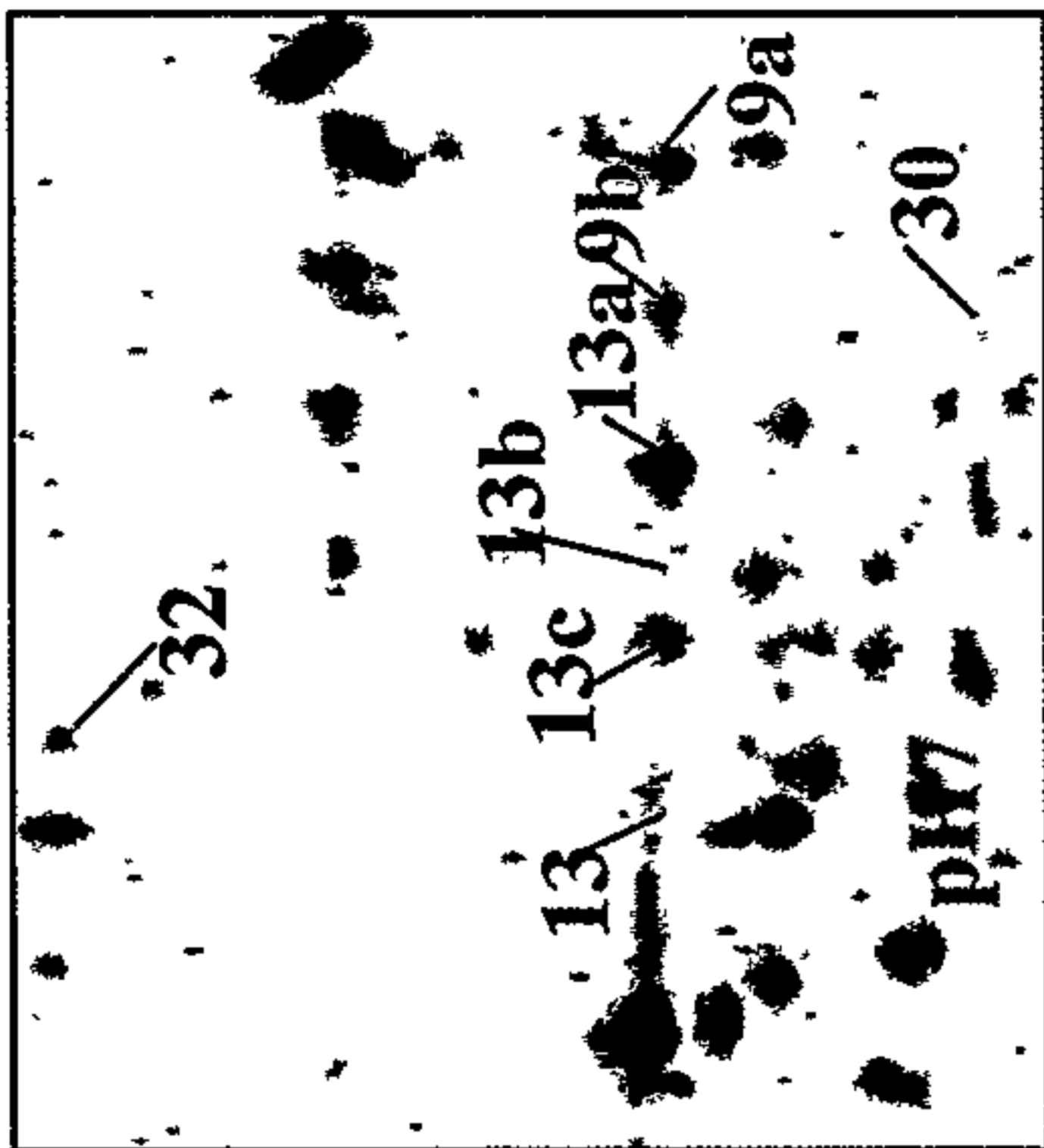
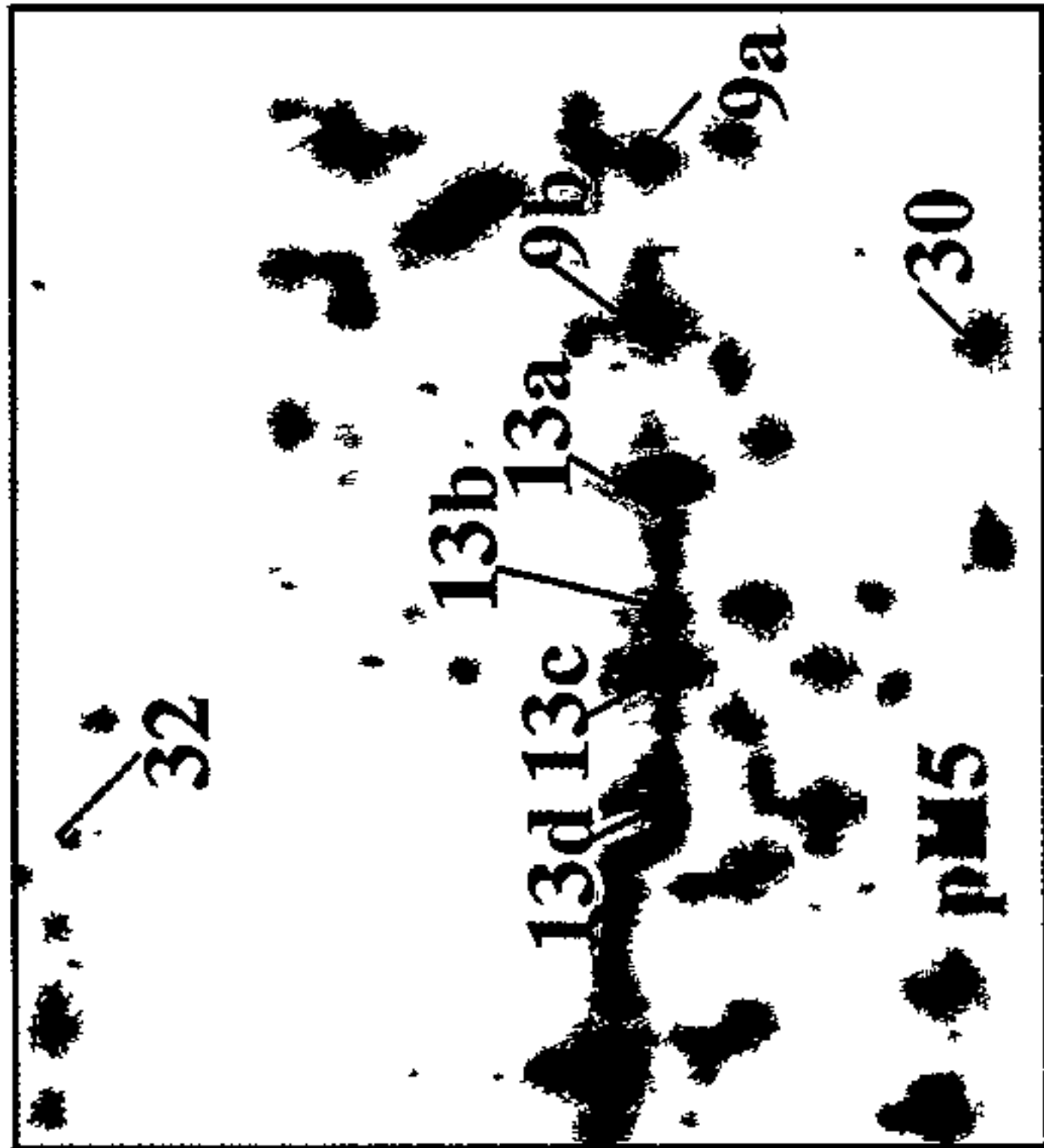
Chemostat 2



Chemostat 3

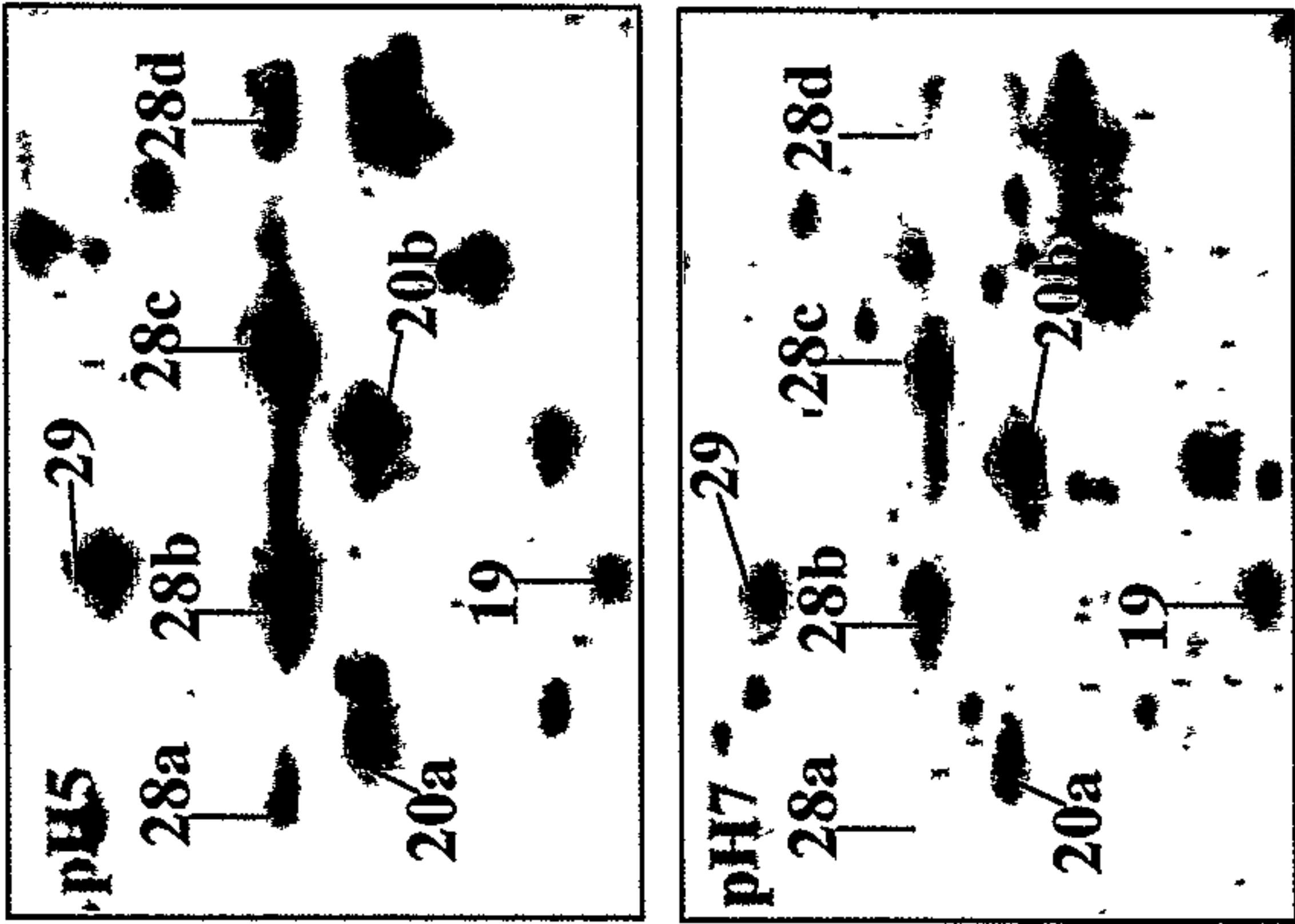


4.6.2

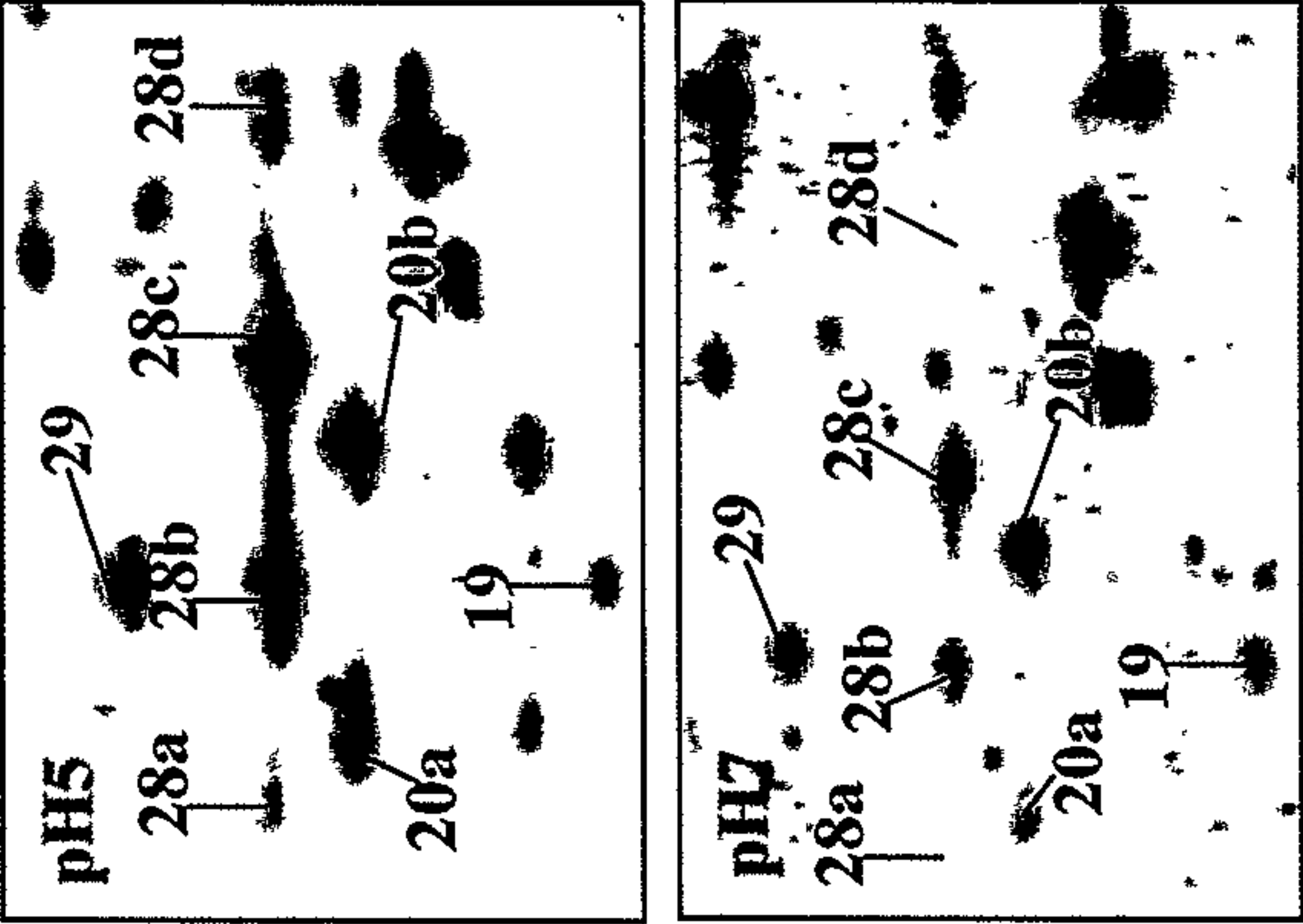


4.6C (cont)

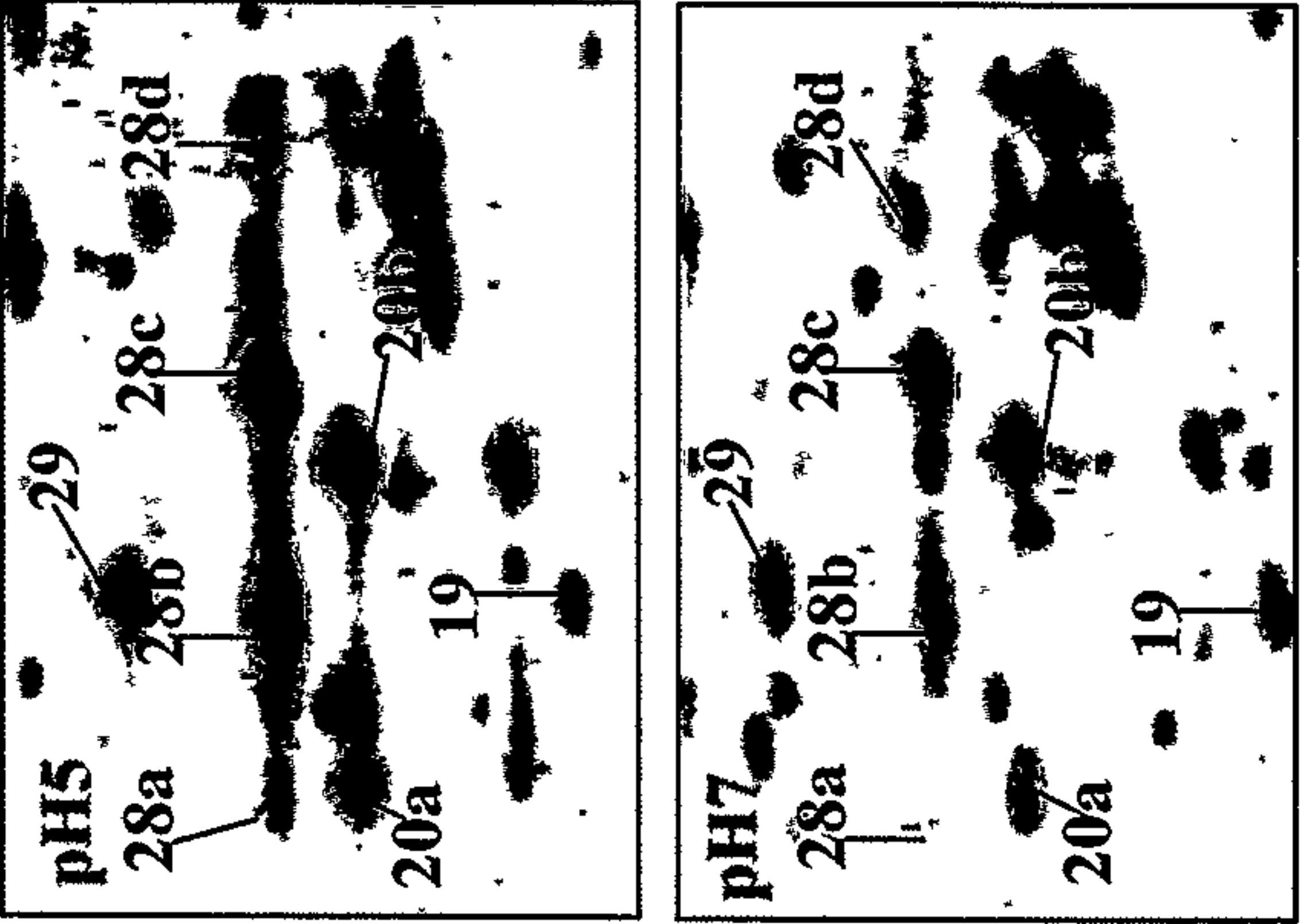
4.6.3 Chemostat 1



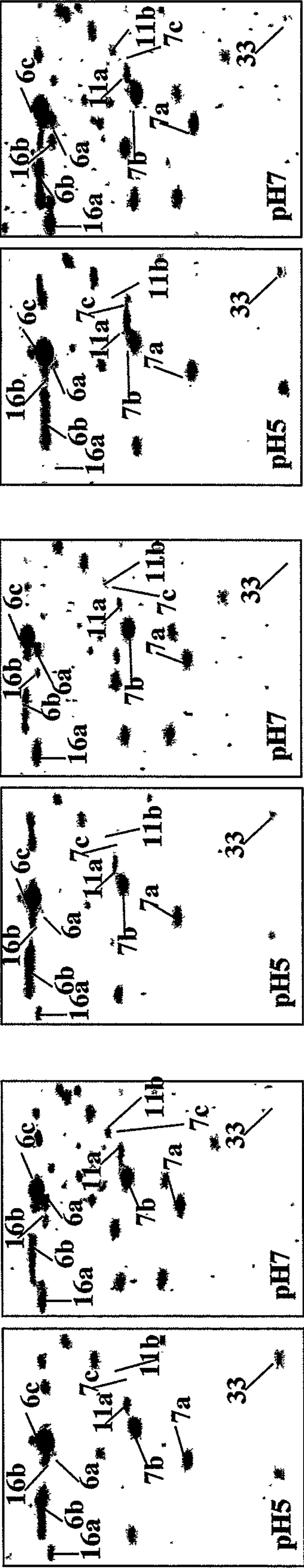
Chemostat 2



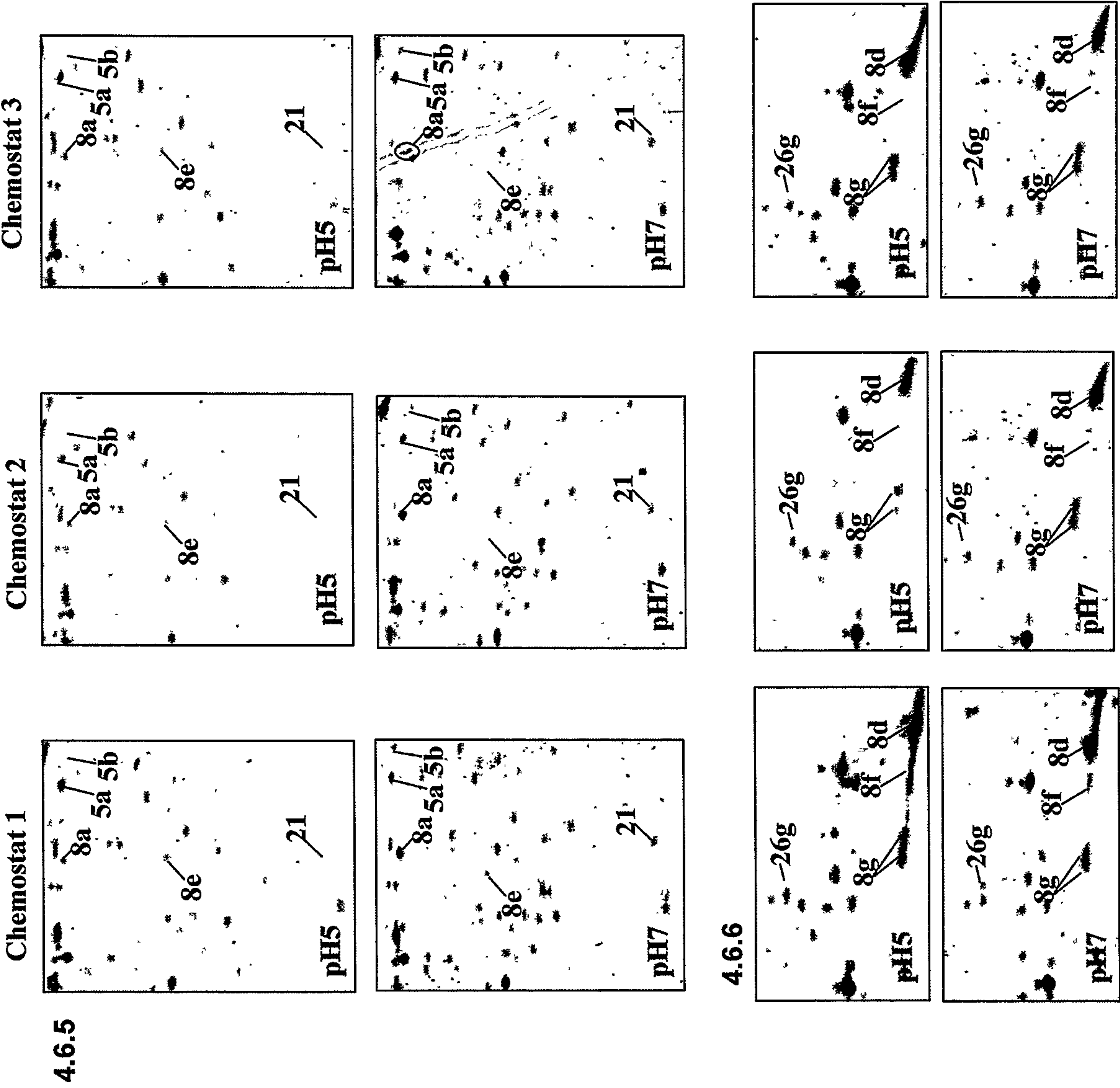
Chemostat 3



4.6.4



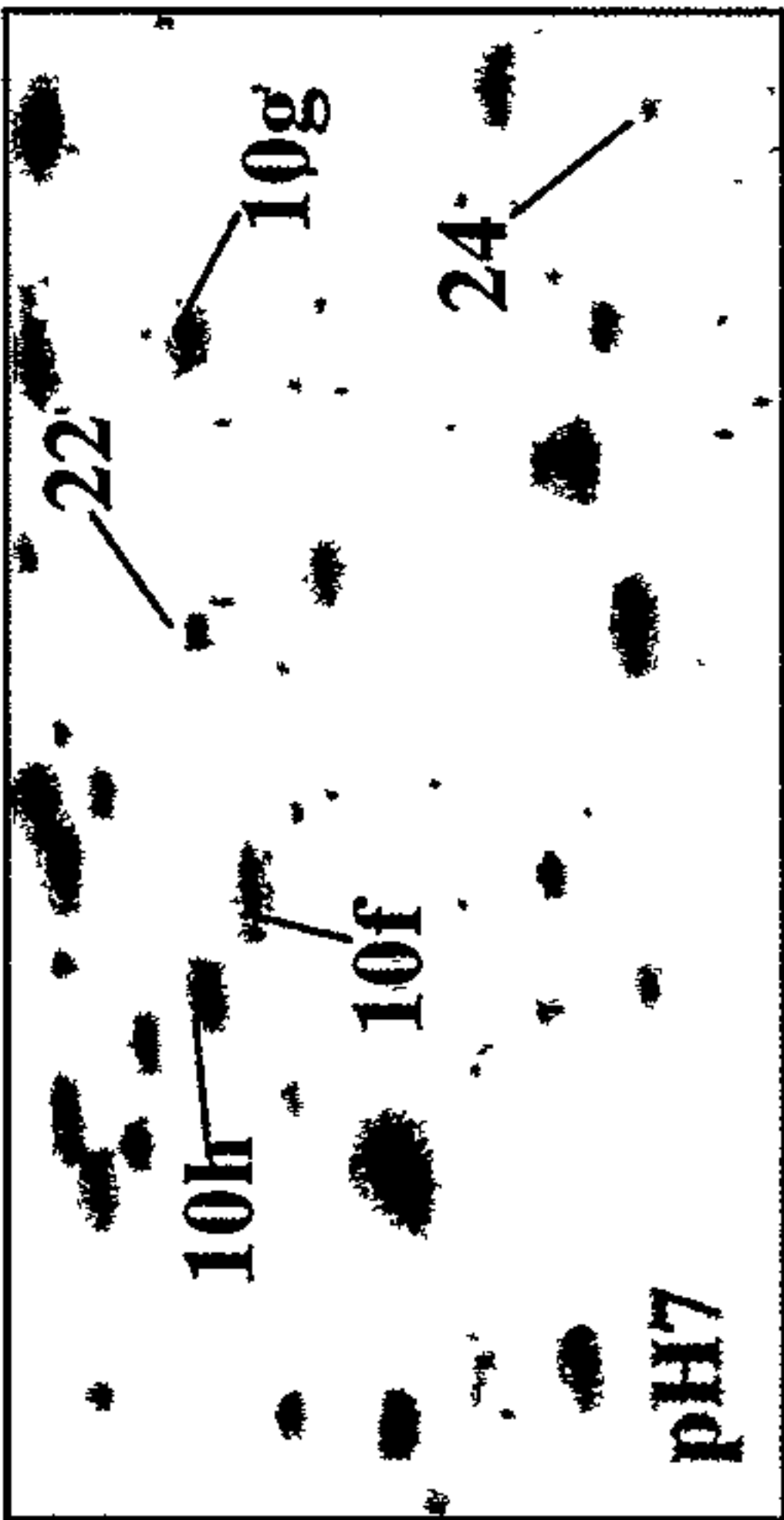
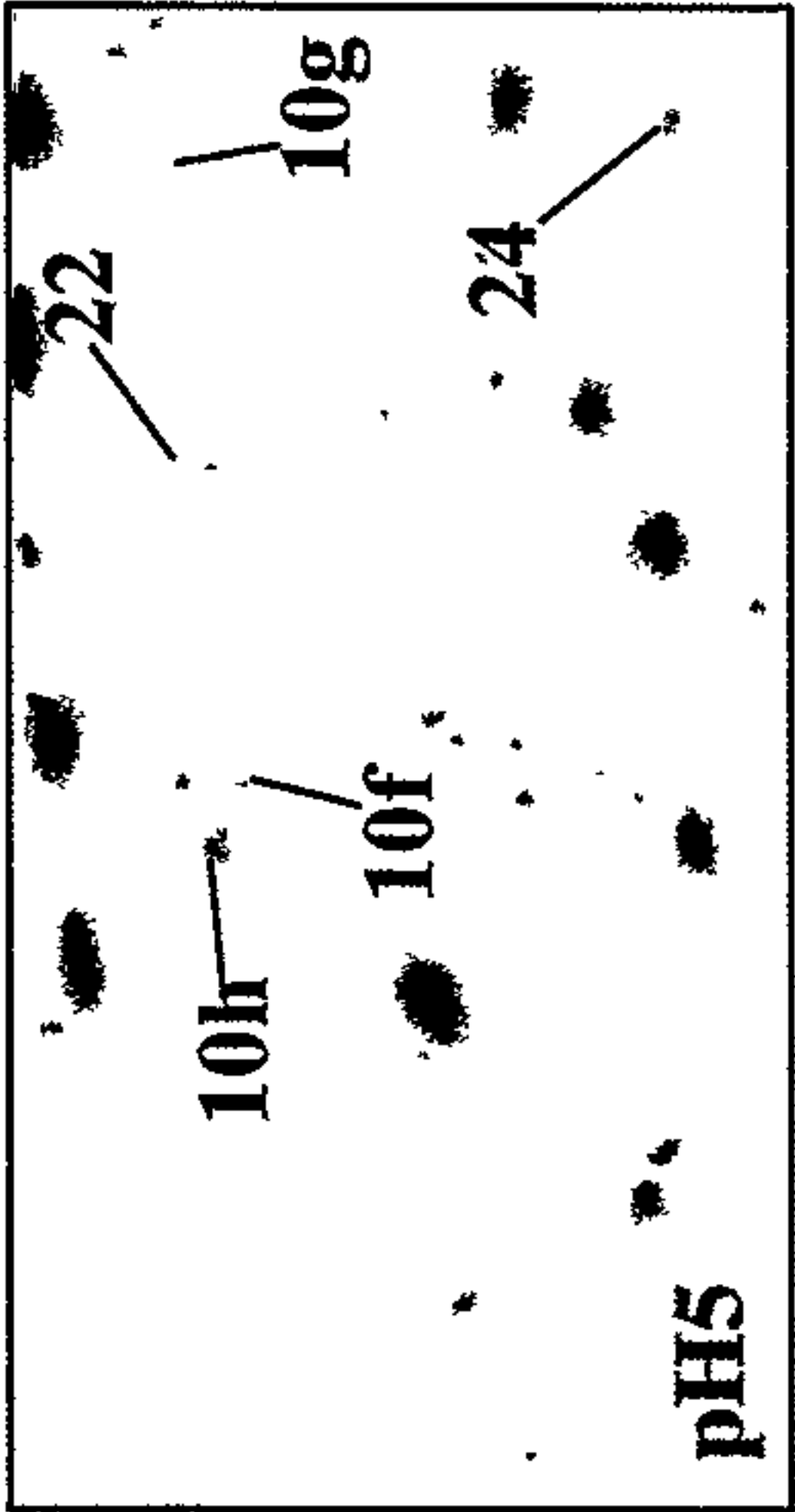
4.6C (cont)



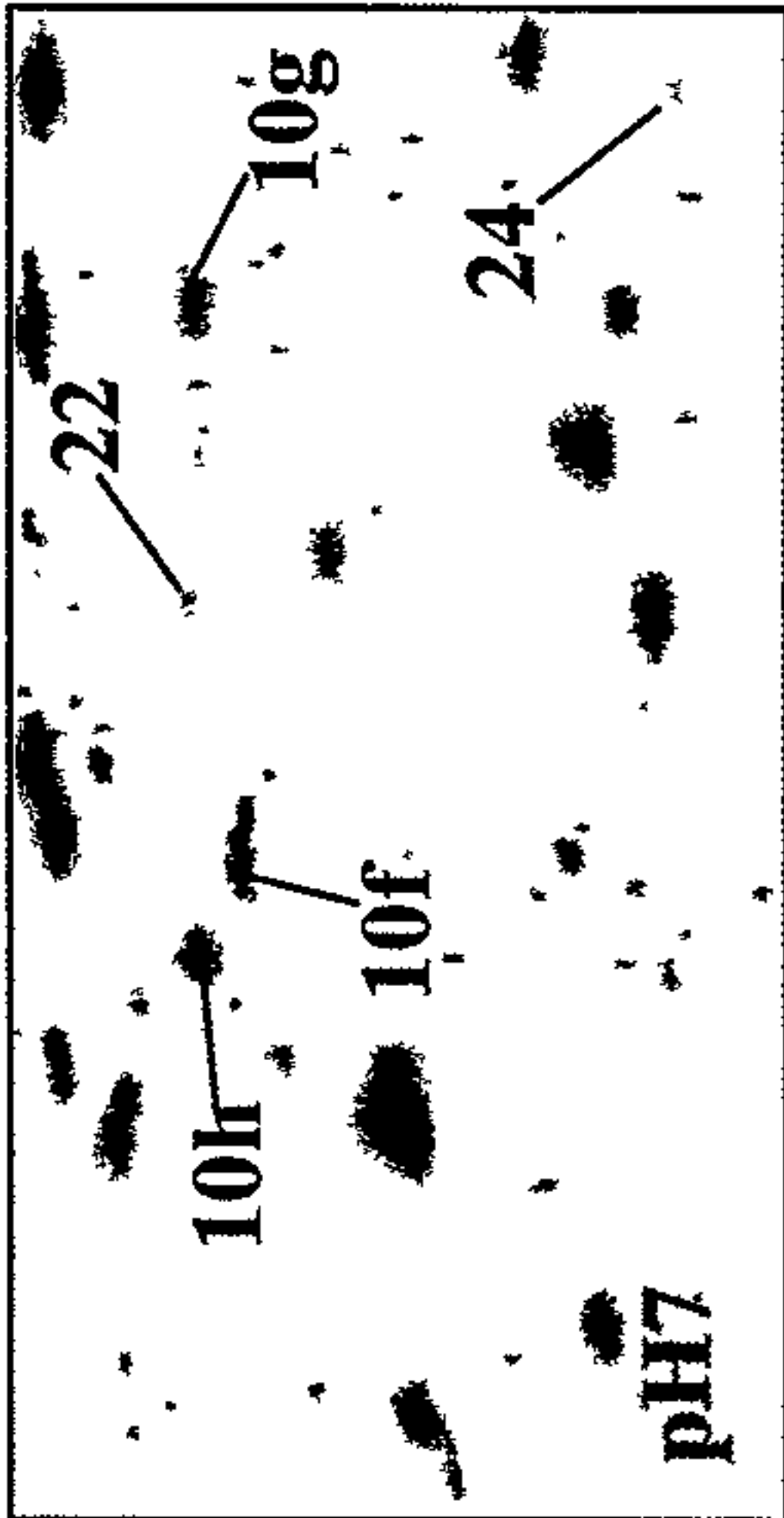
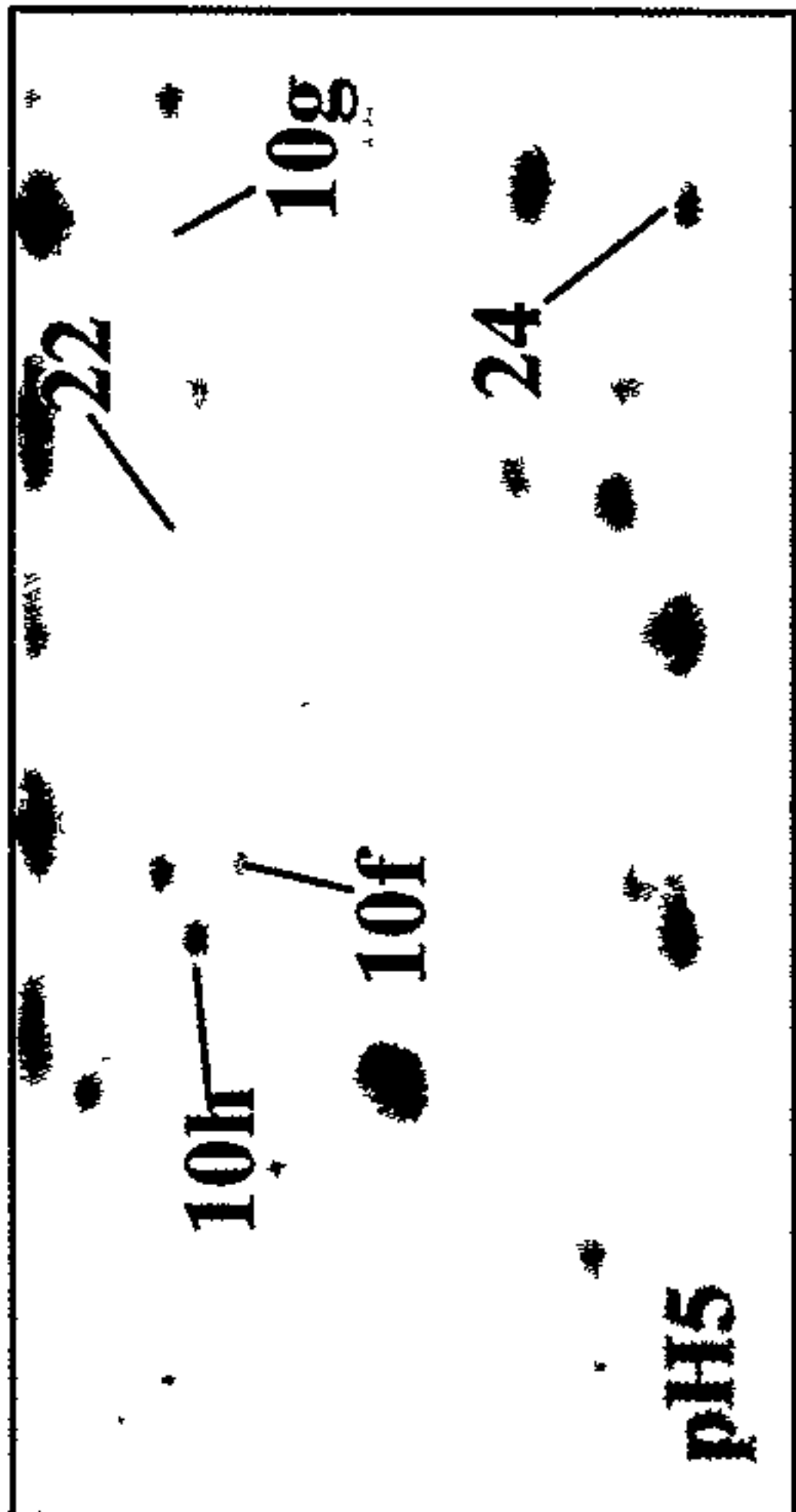
4.6C (cont)

4.6.7

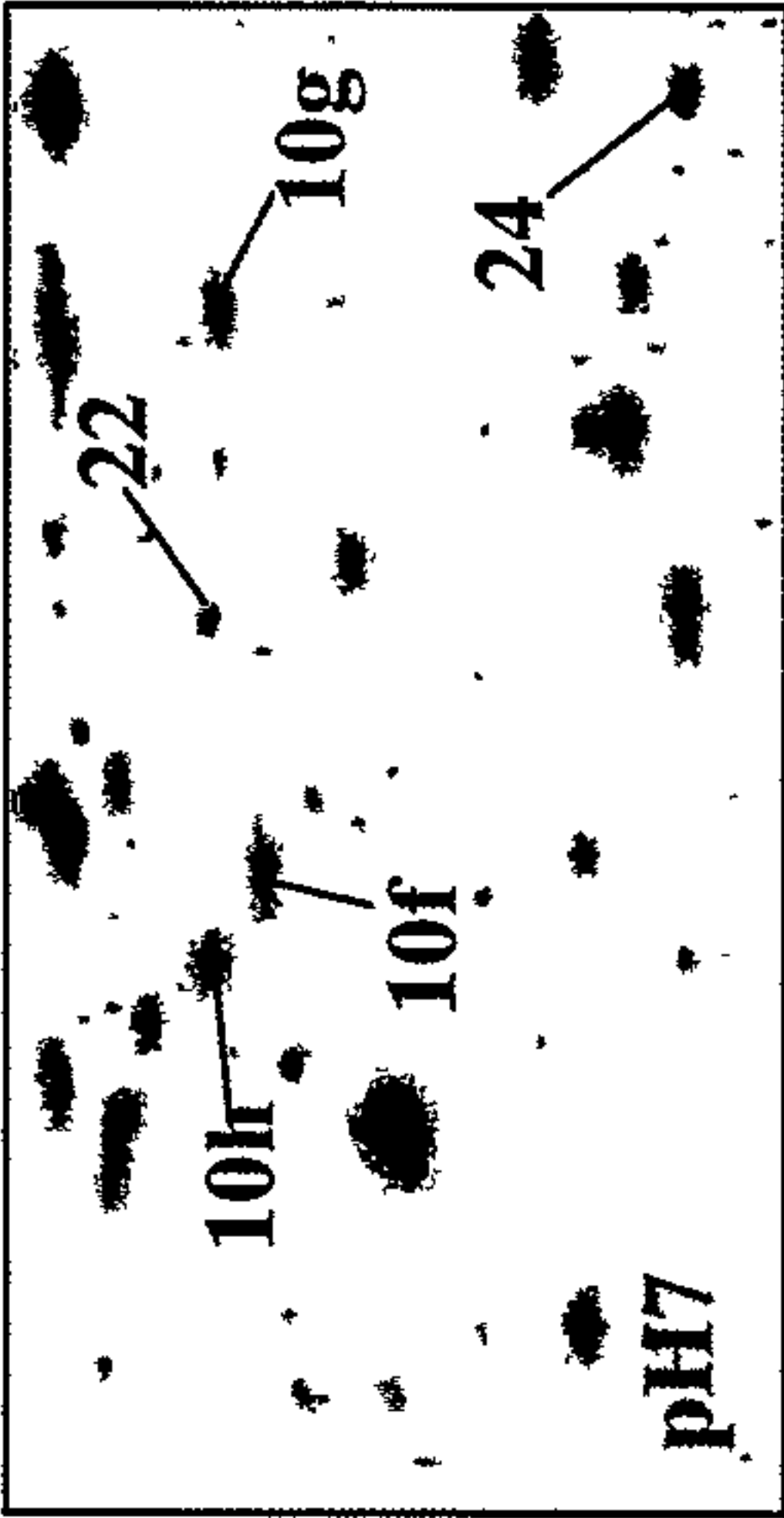
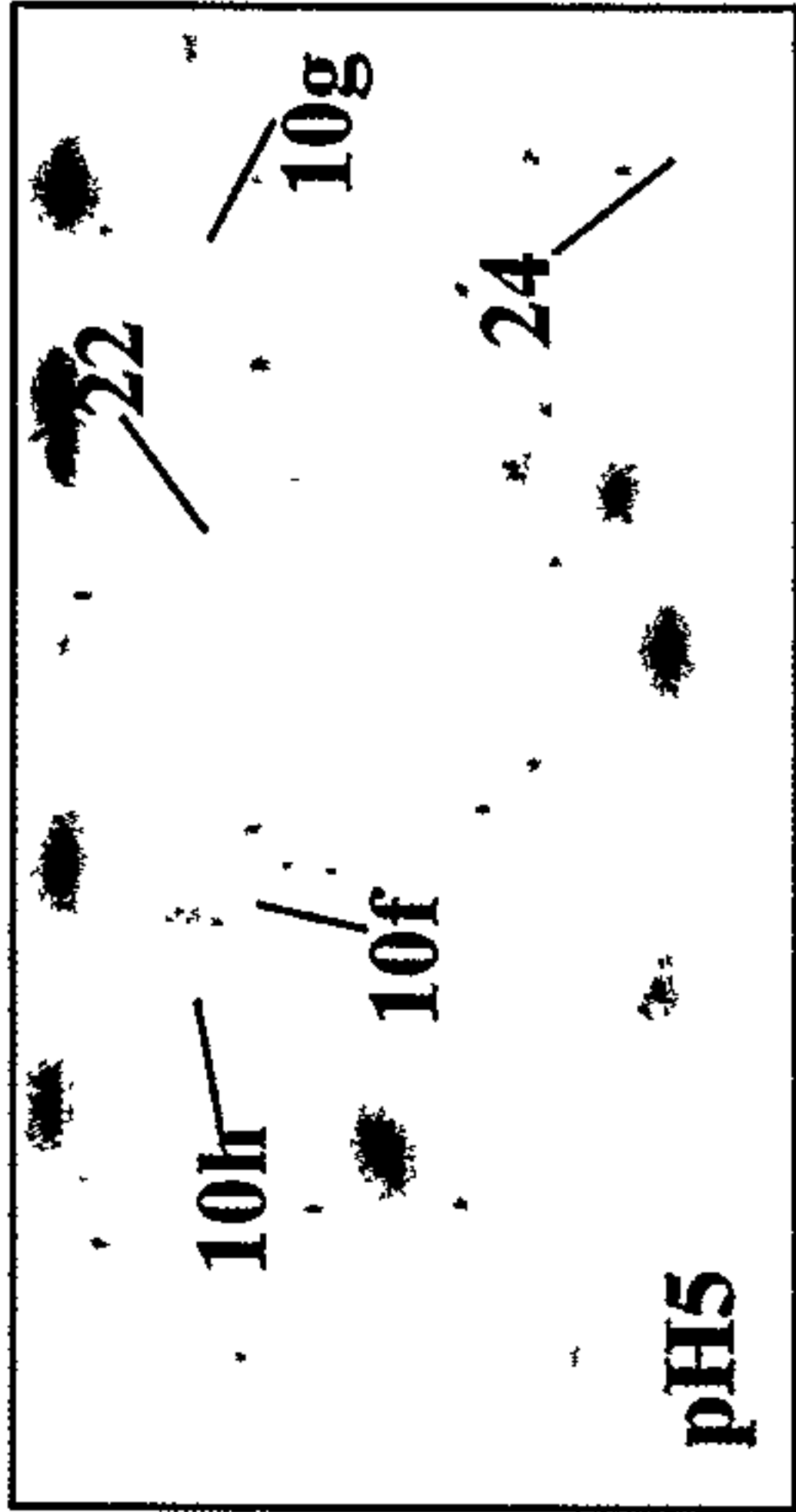
Chemostat 1



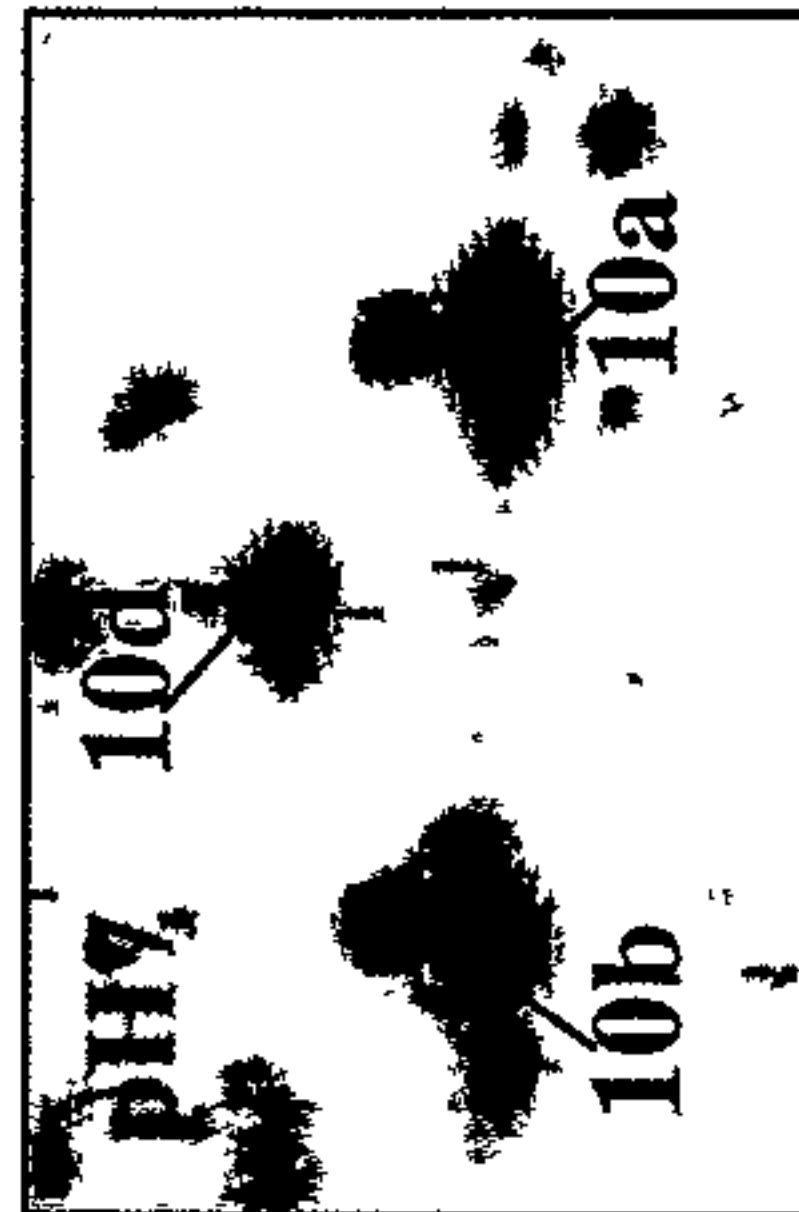
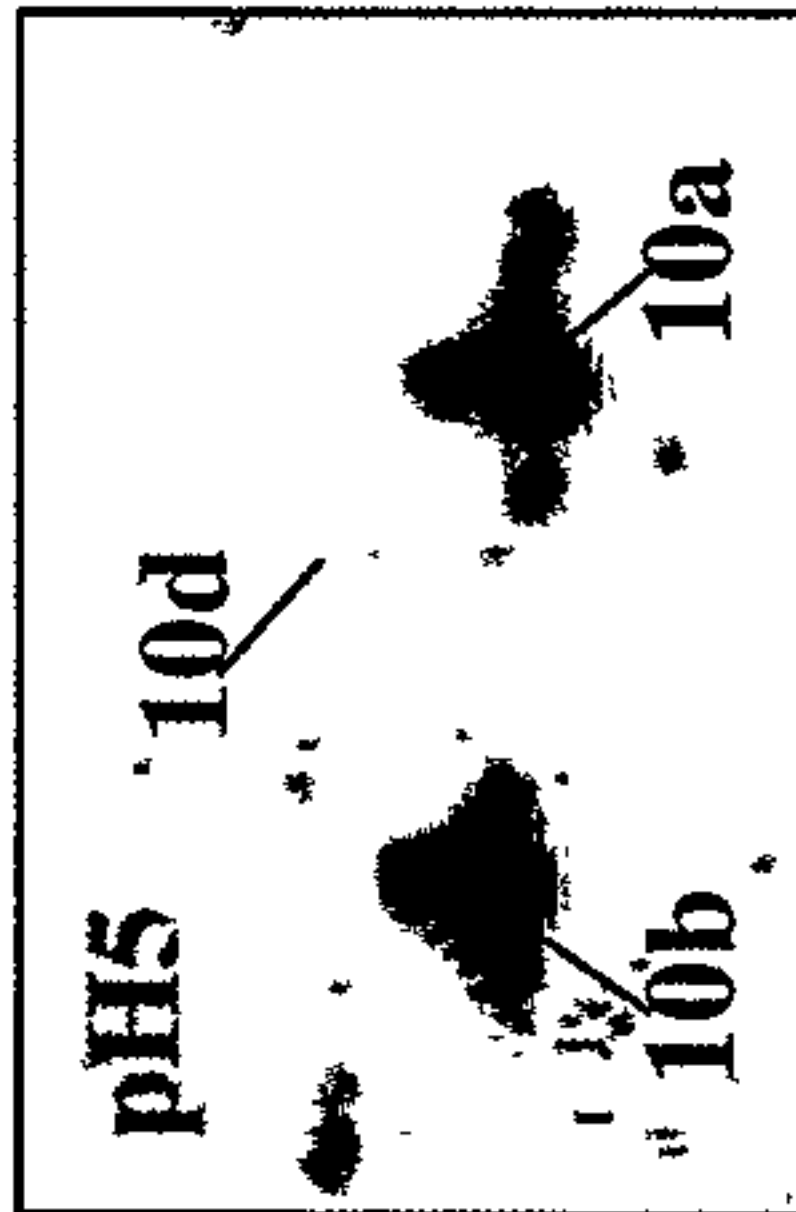
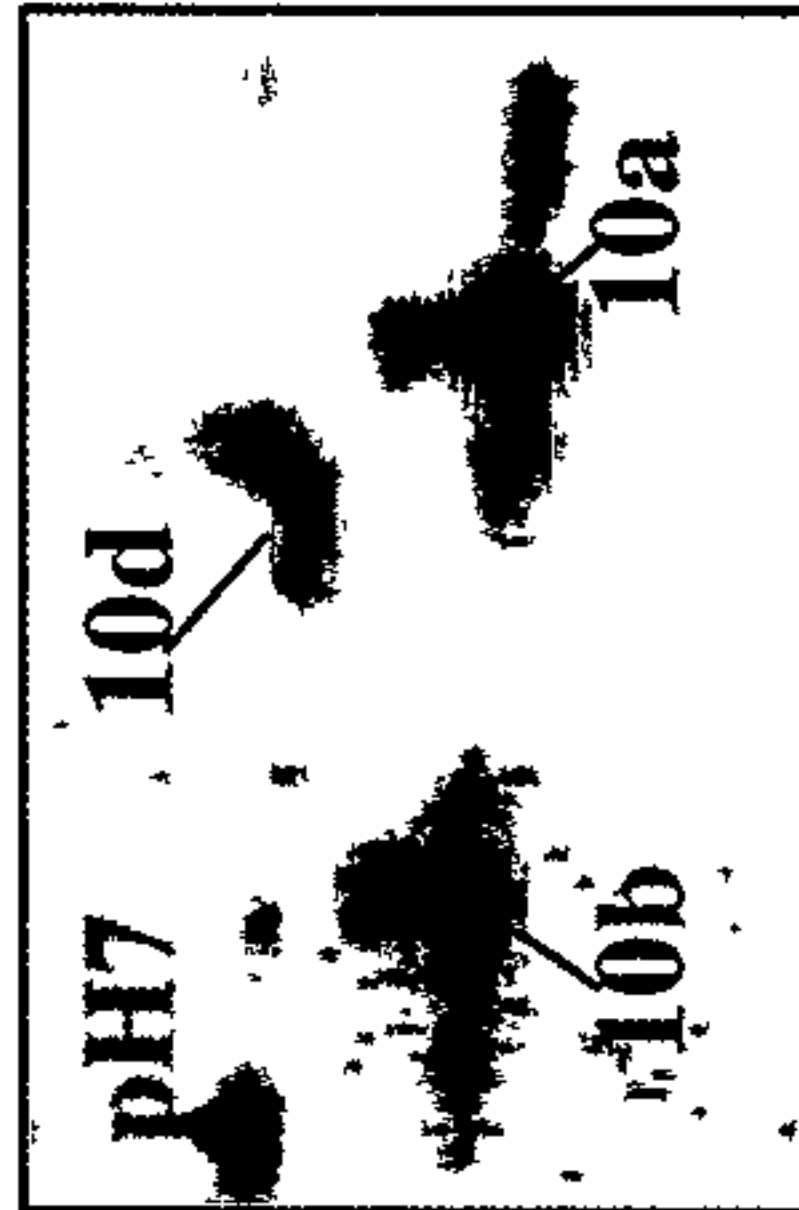
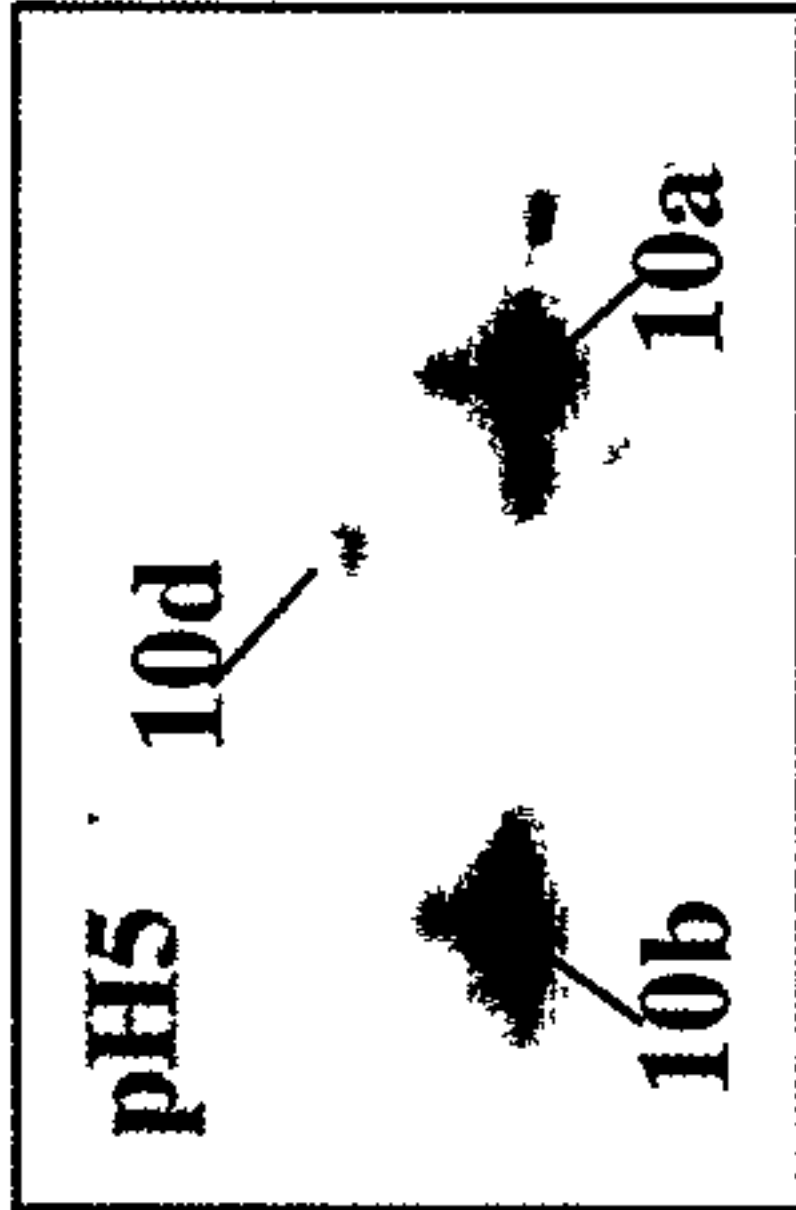
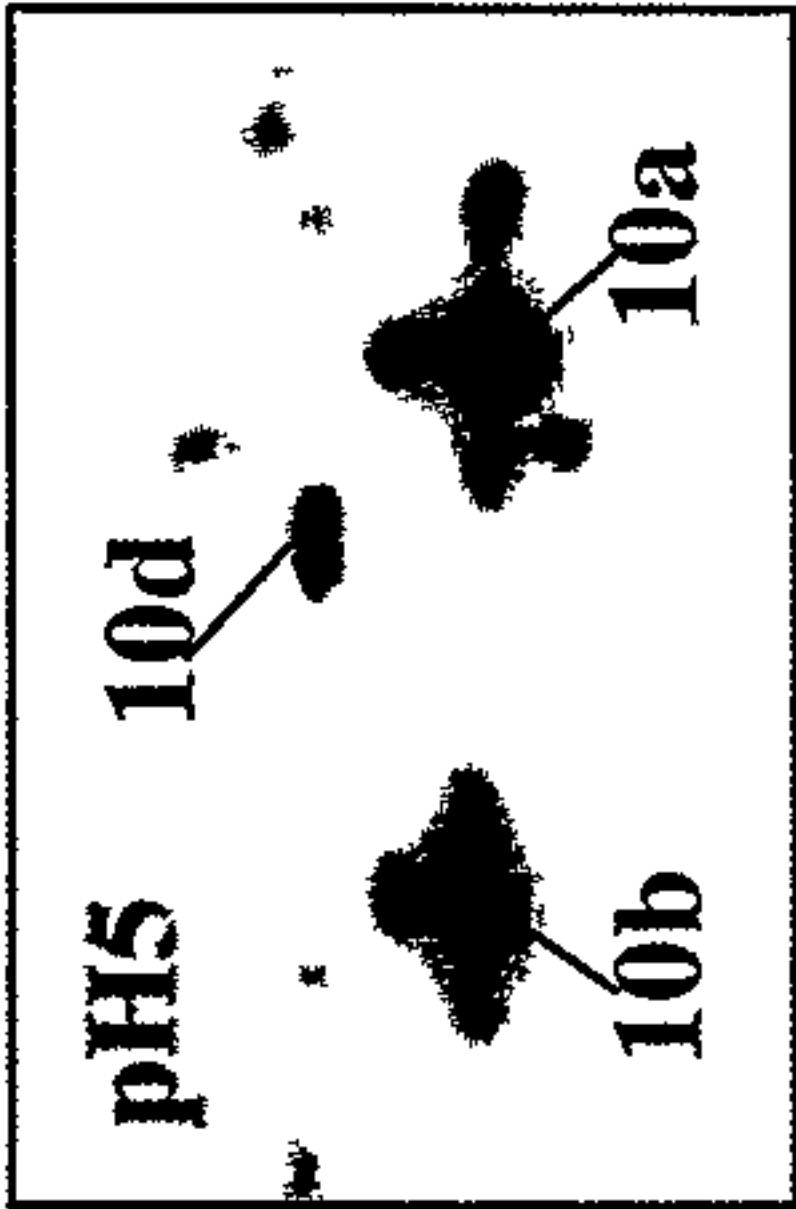
Chemostat 2



Chemostat 3



4.6.8



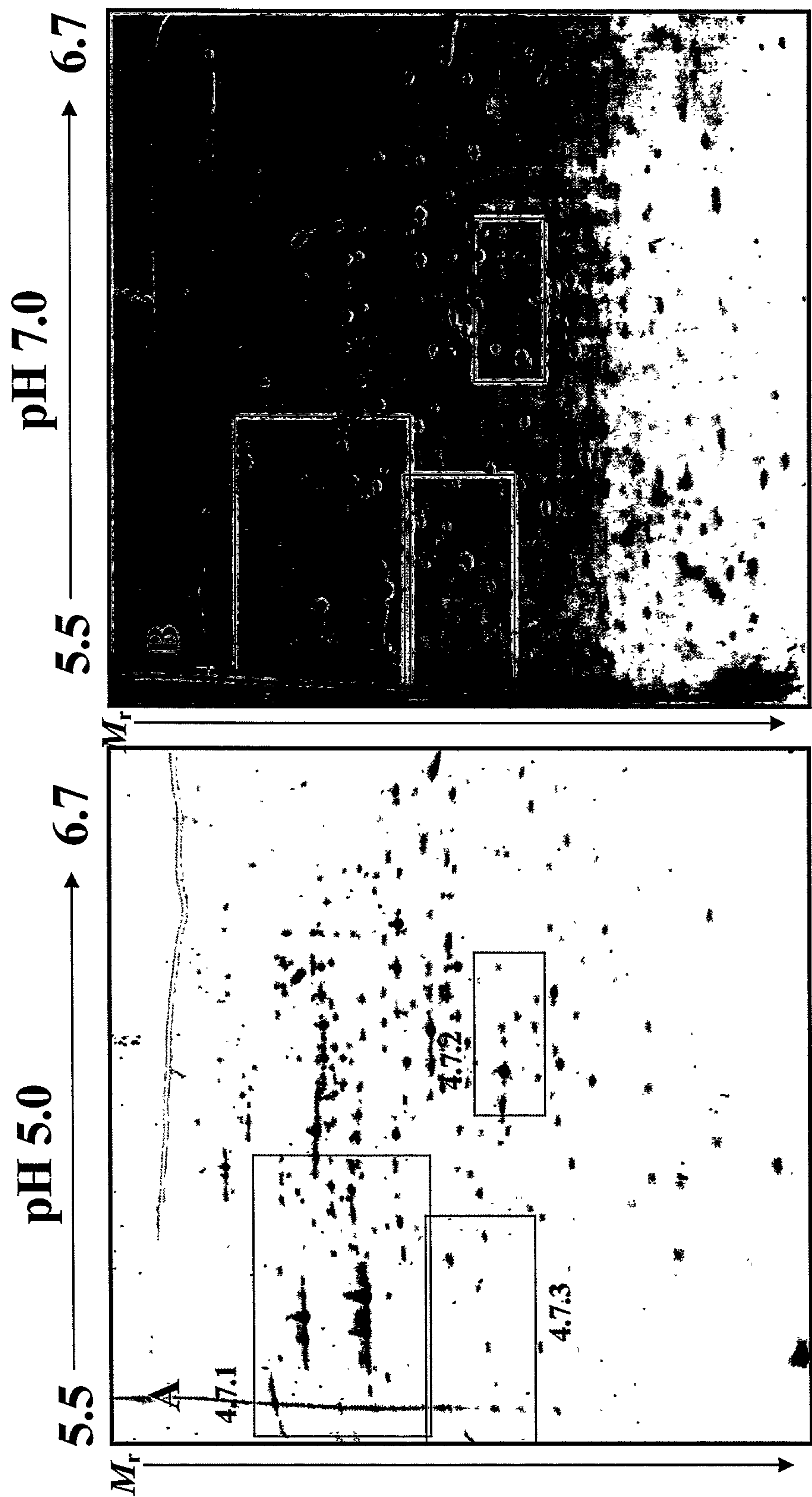
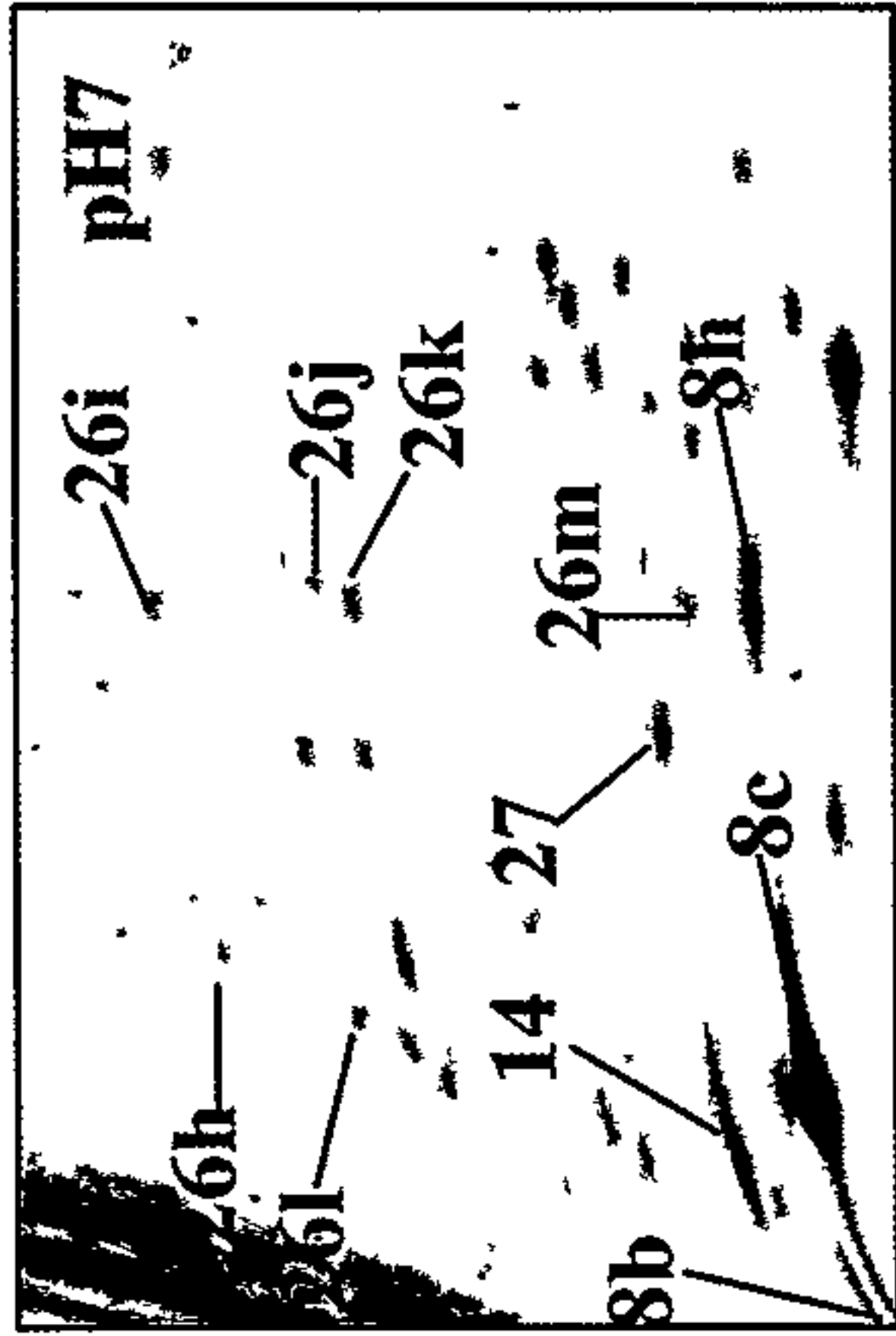
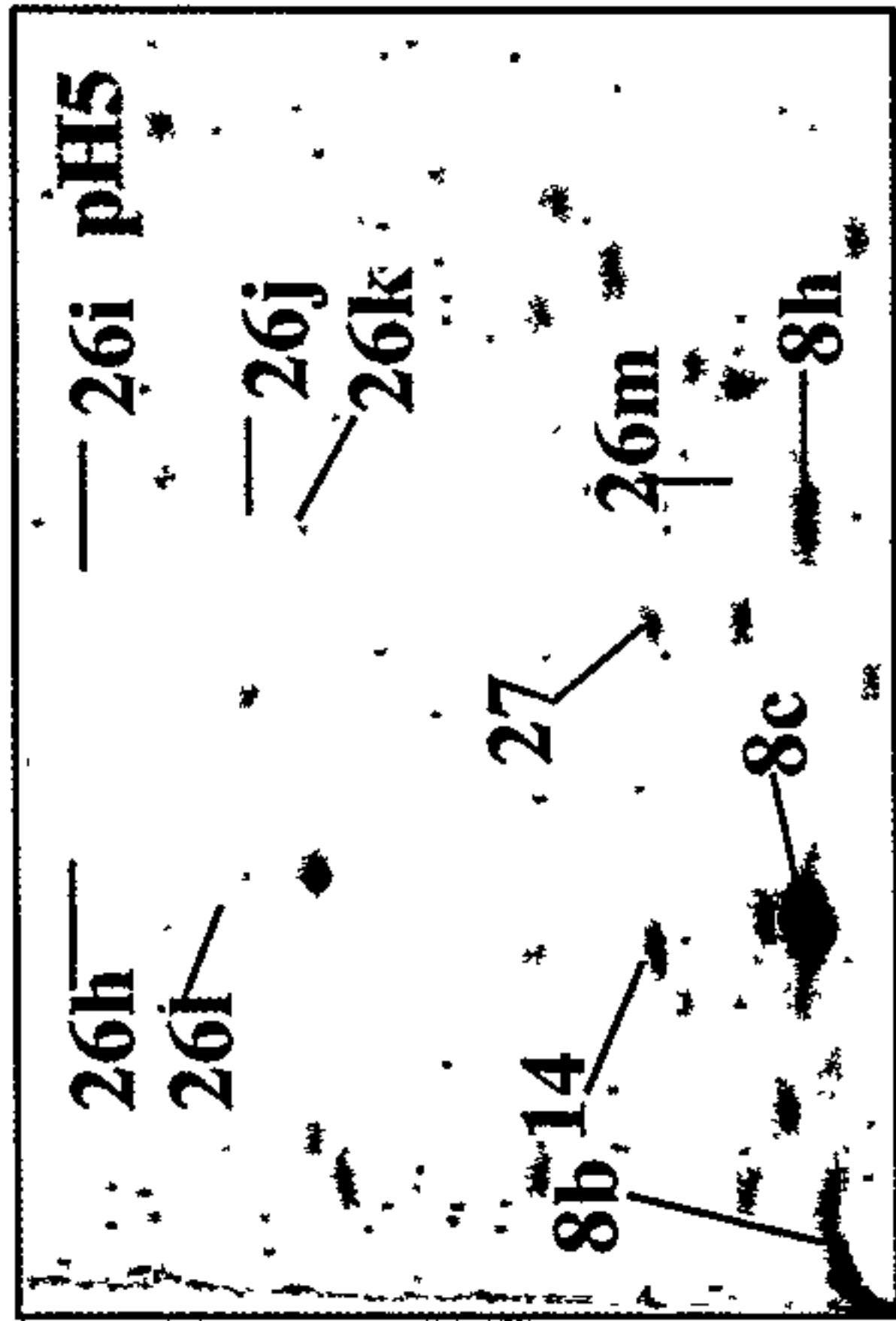


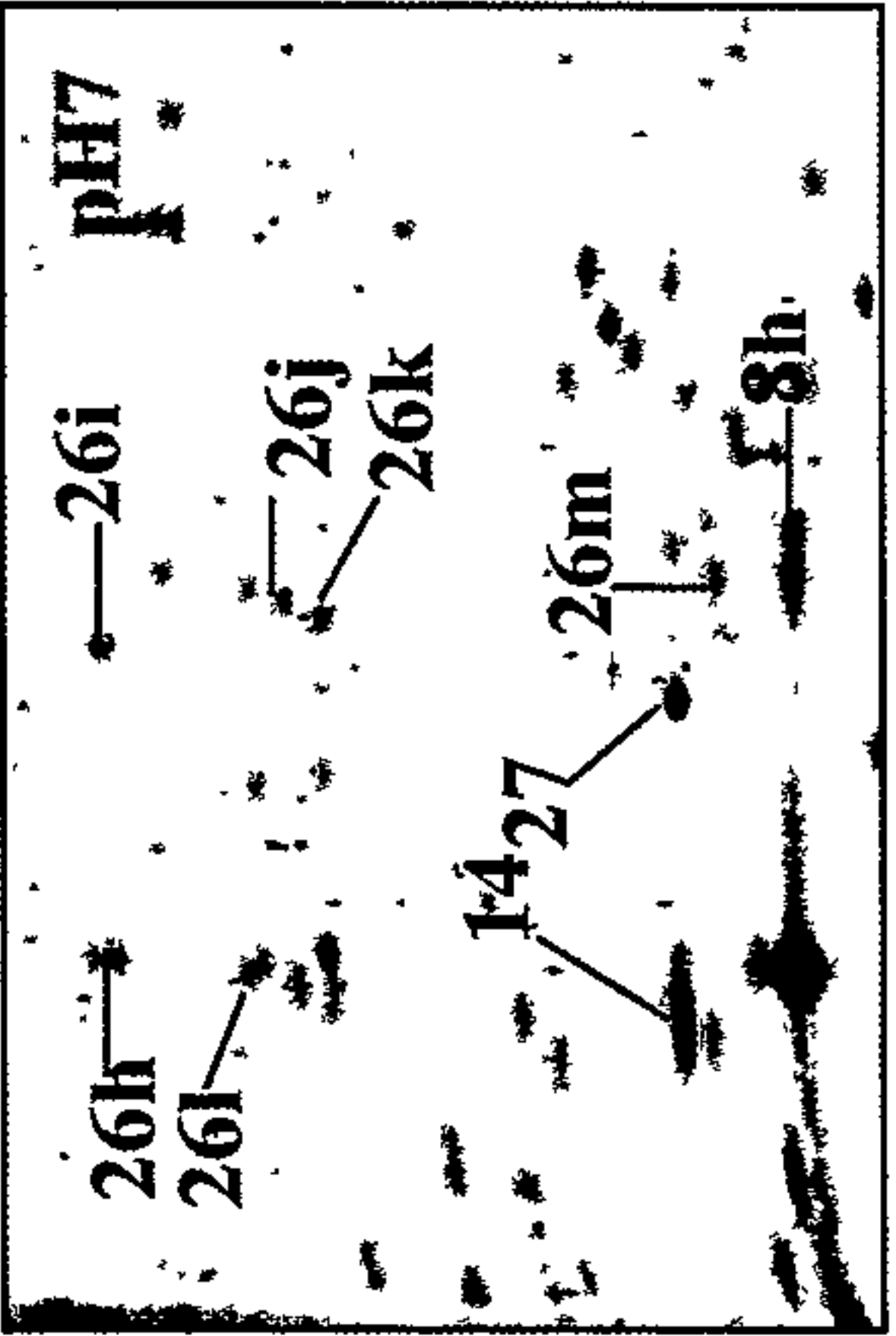
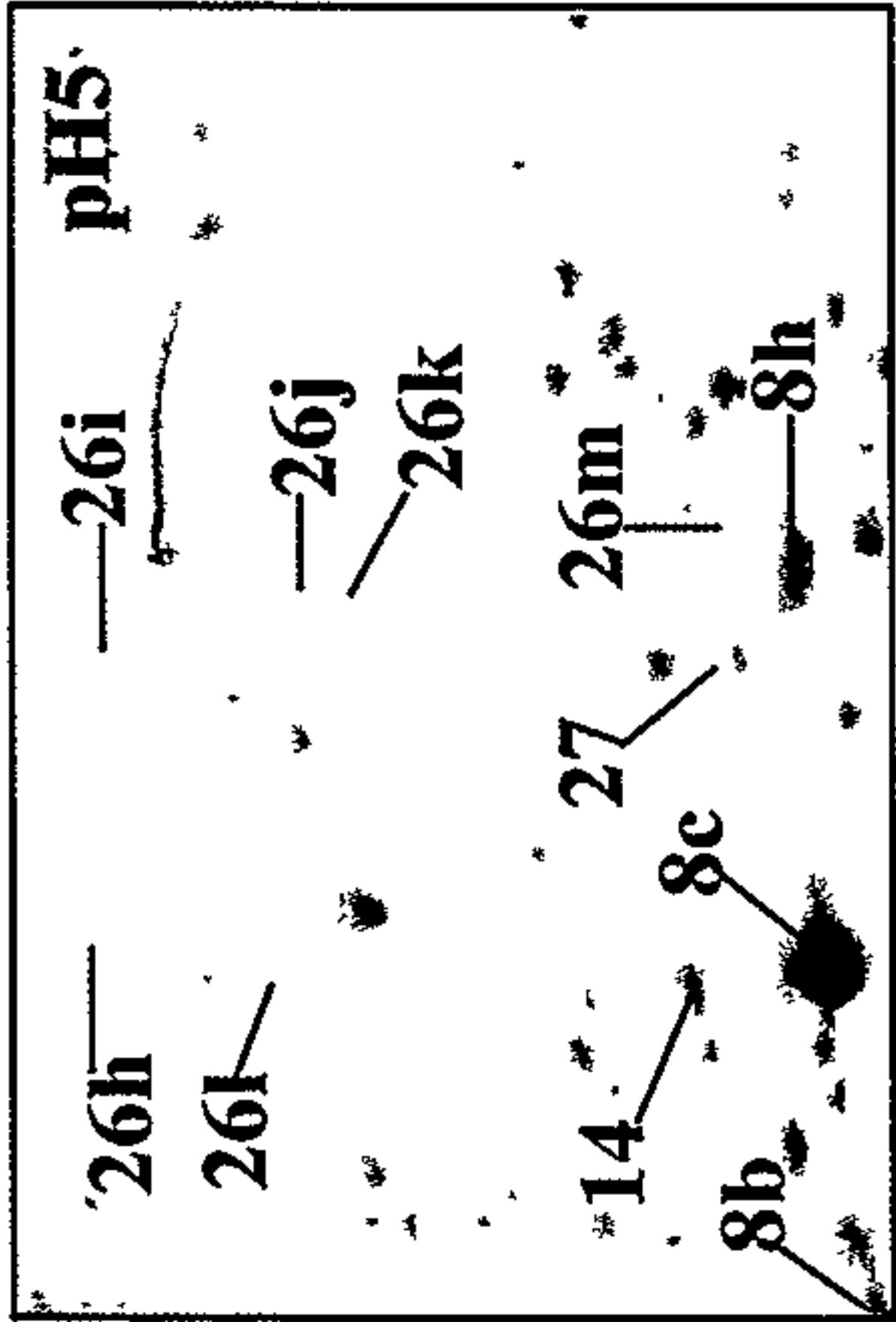
Figure 4.7. 2-DGE Sub-proteome maps (pH 5.5 – 6.7) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas (4.7.1, 4.7.2, and 4.7.3) depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed areas, corresponding to their respective chemostat 2-DGE gels, are illustrated in 4.7C with spot numbers corresponding to those in Table 4.1.

4.7C

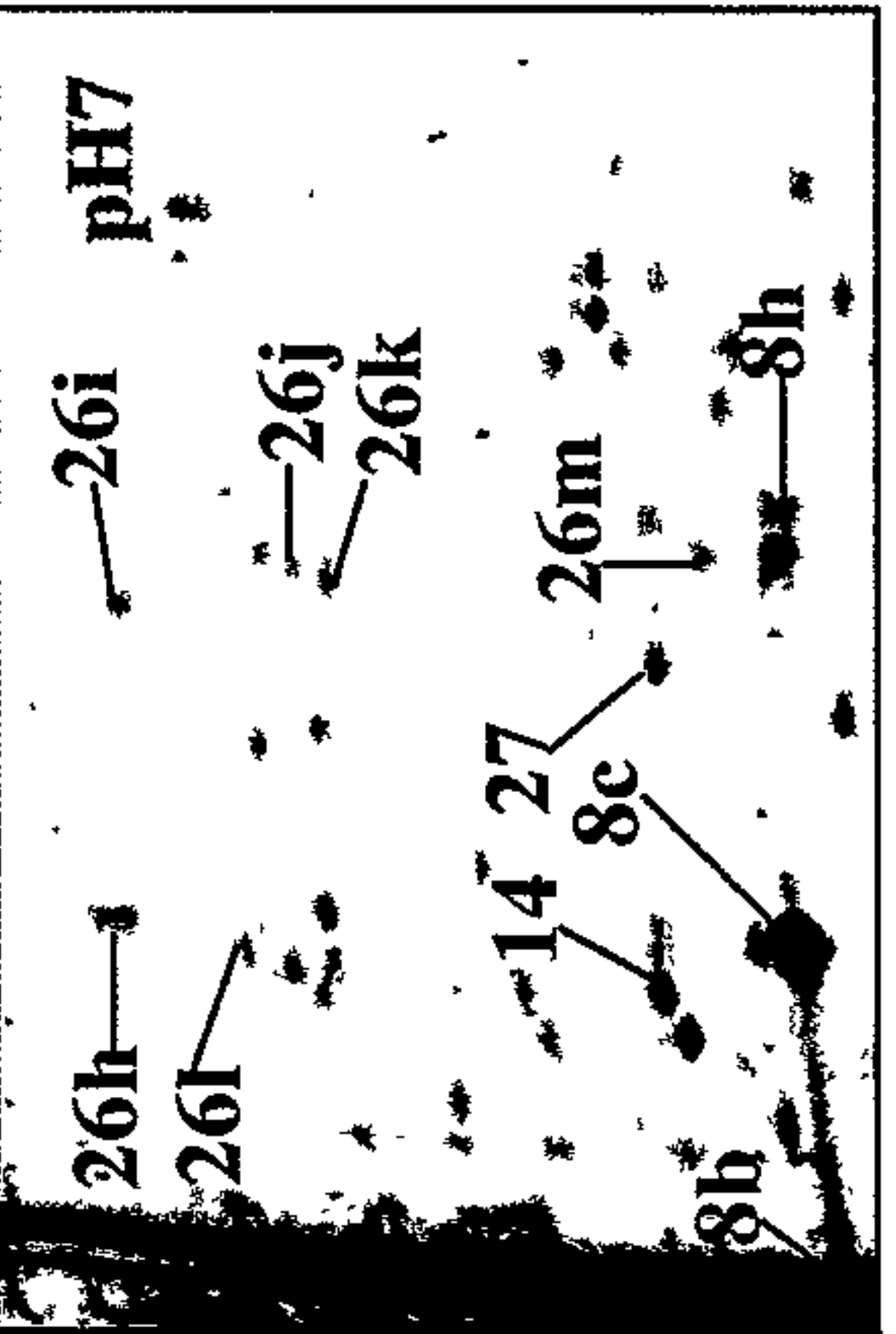
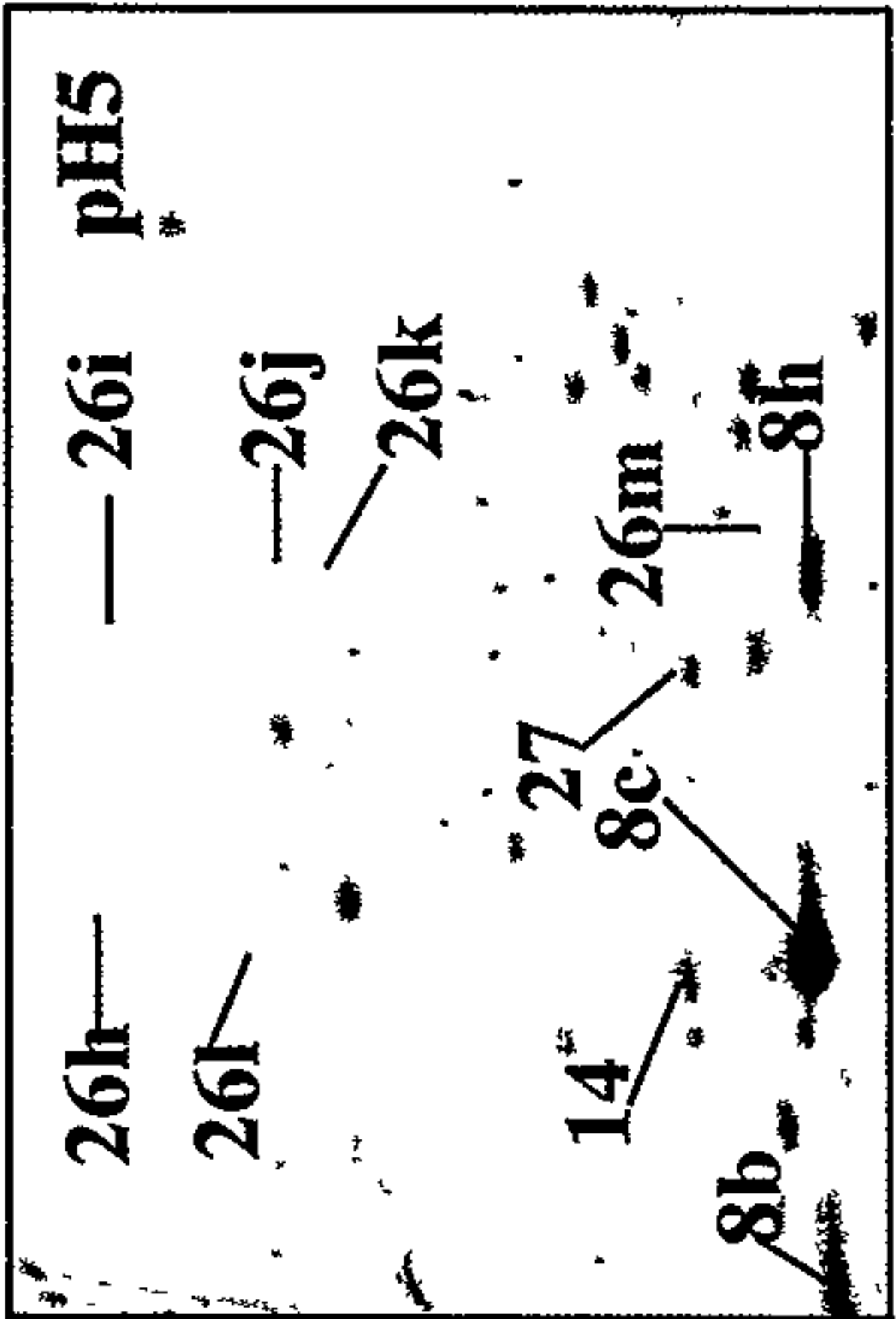
4.7.1 Chemostat 1



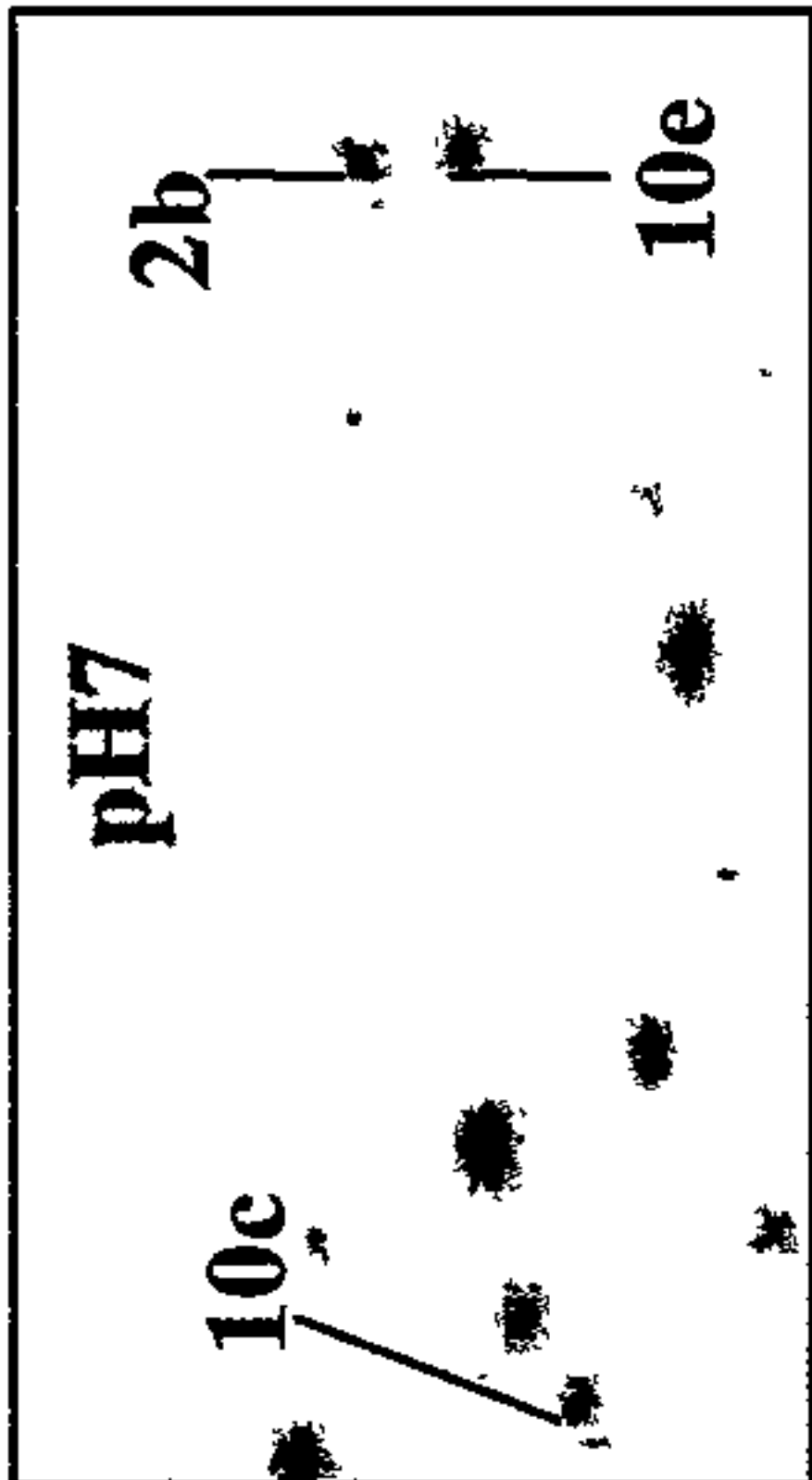
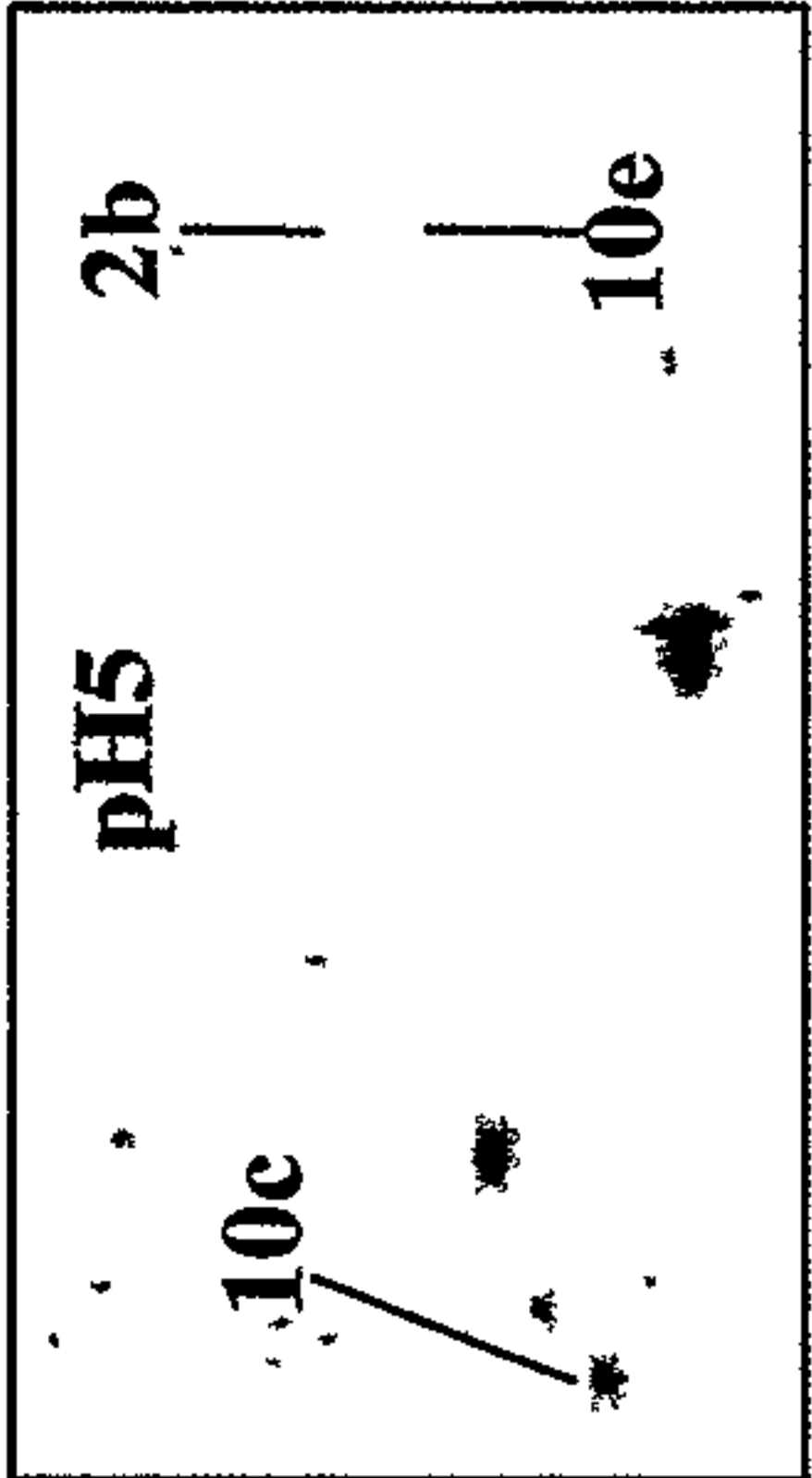
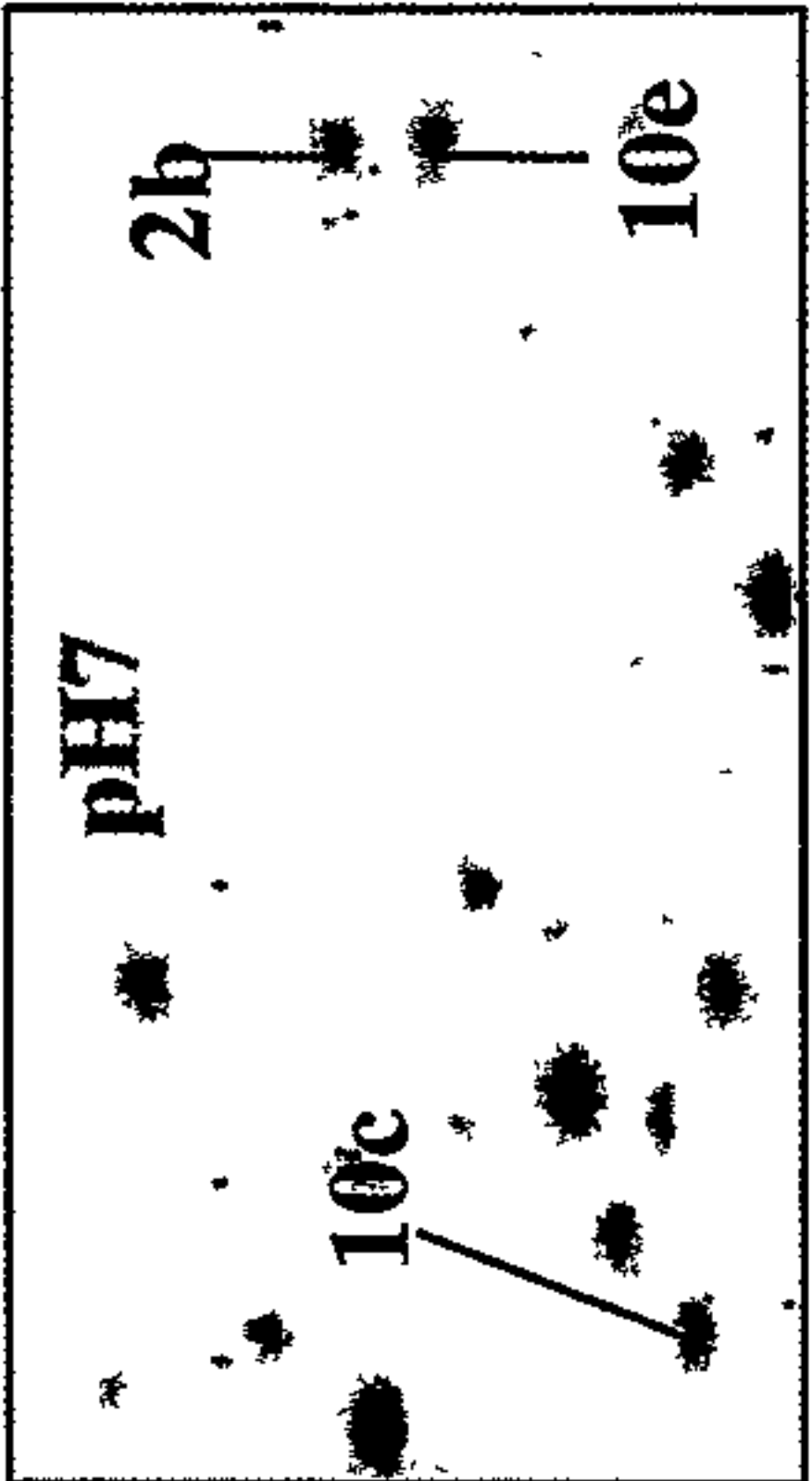
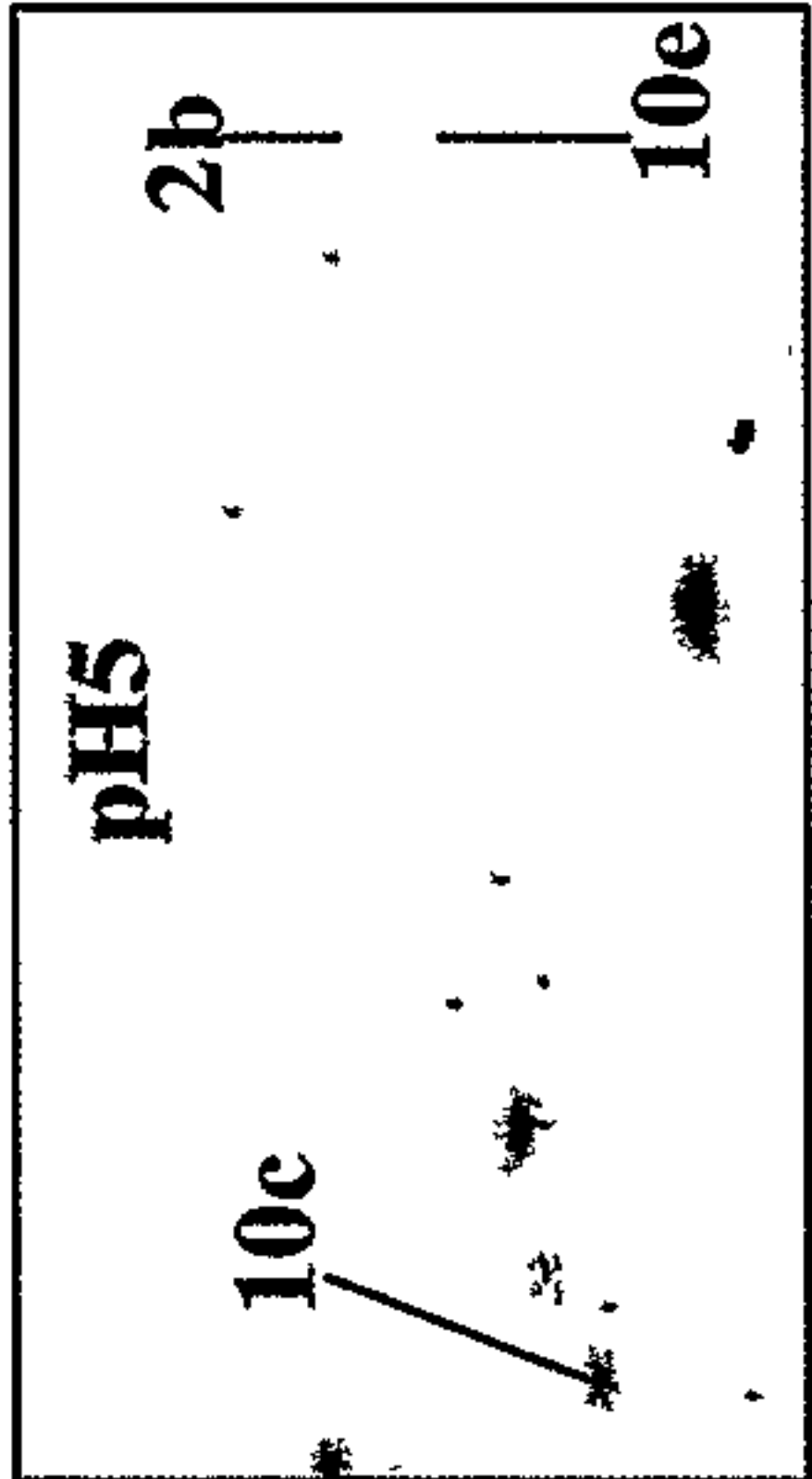
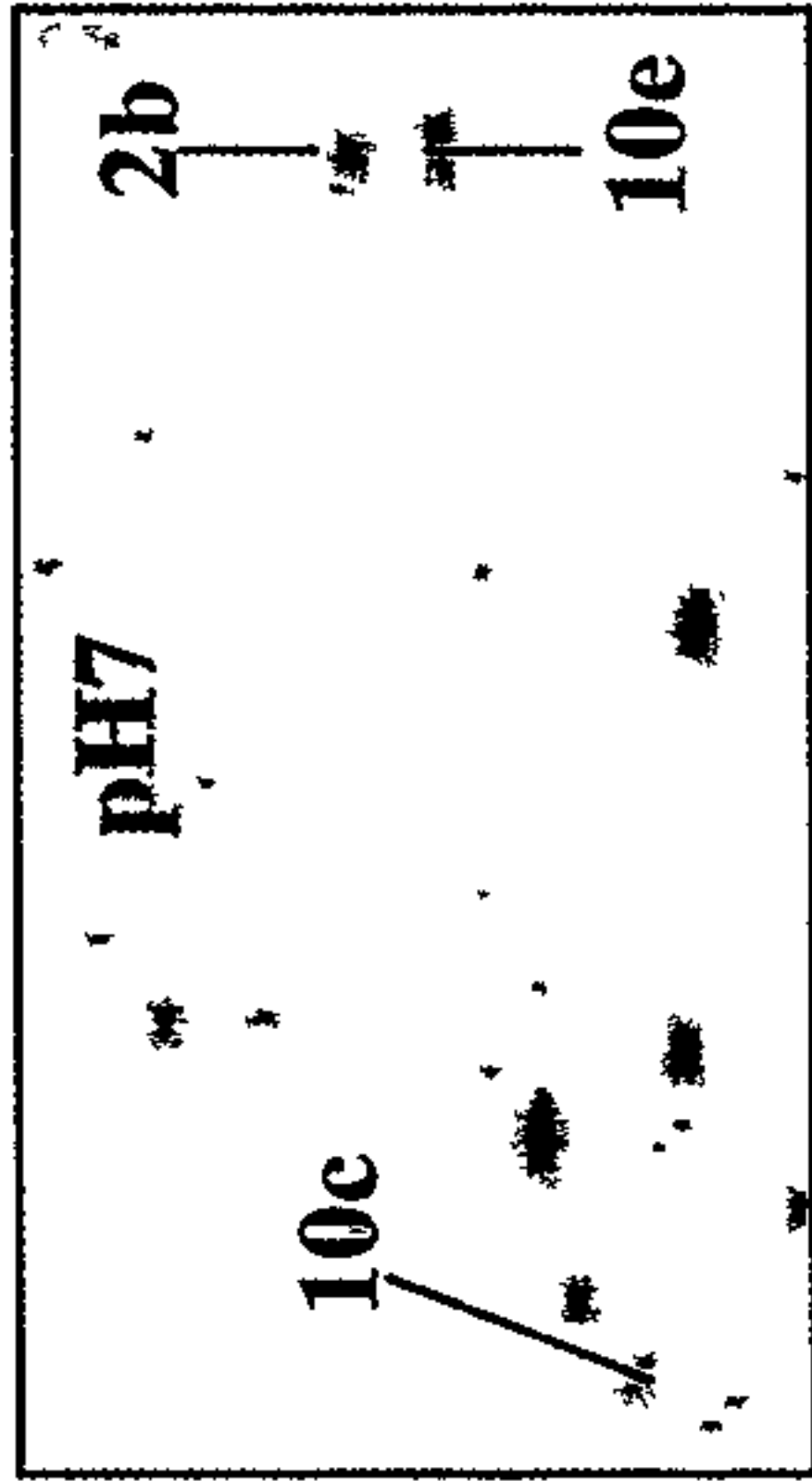
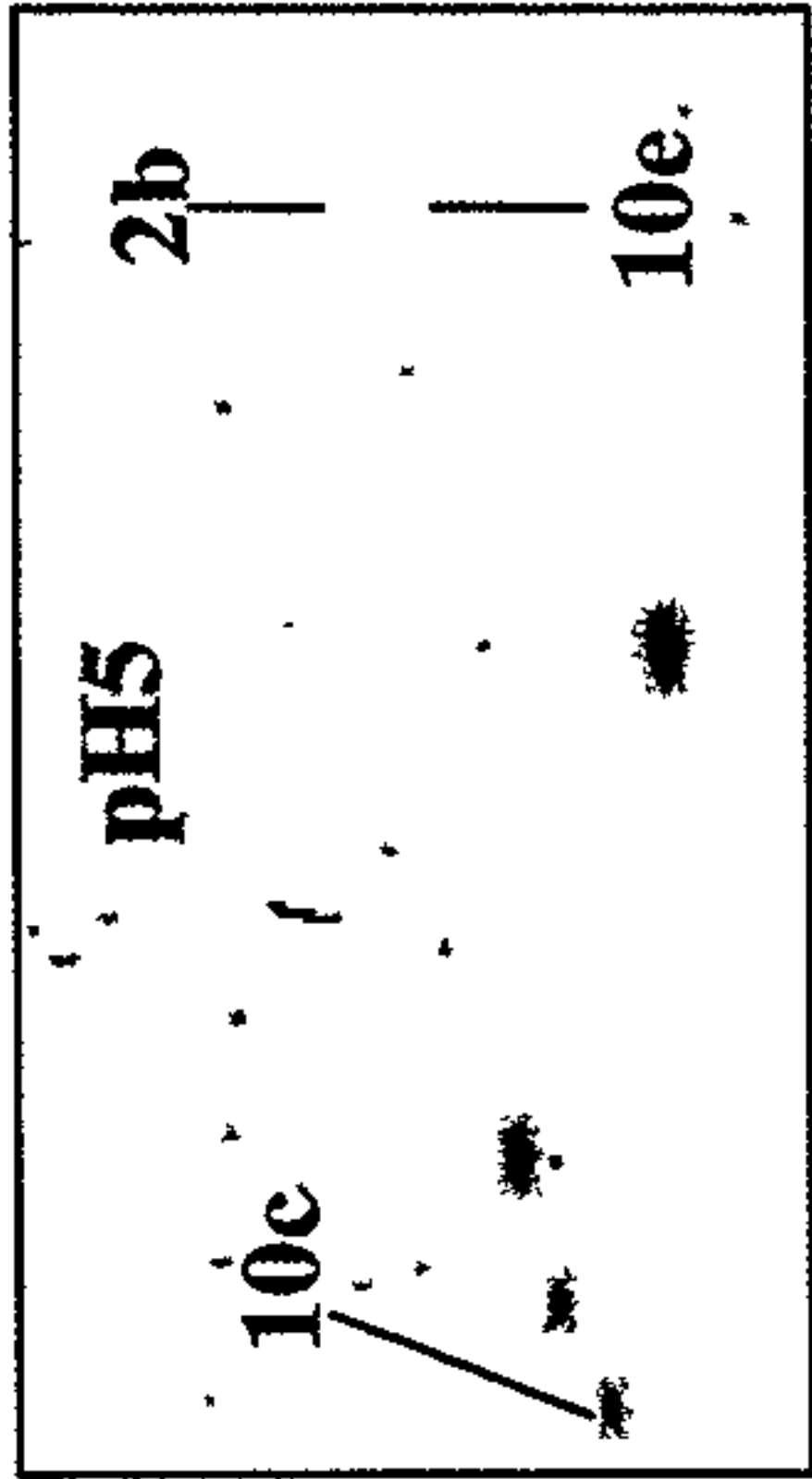
Chemostat 2



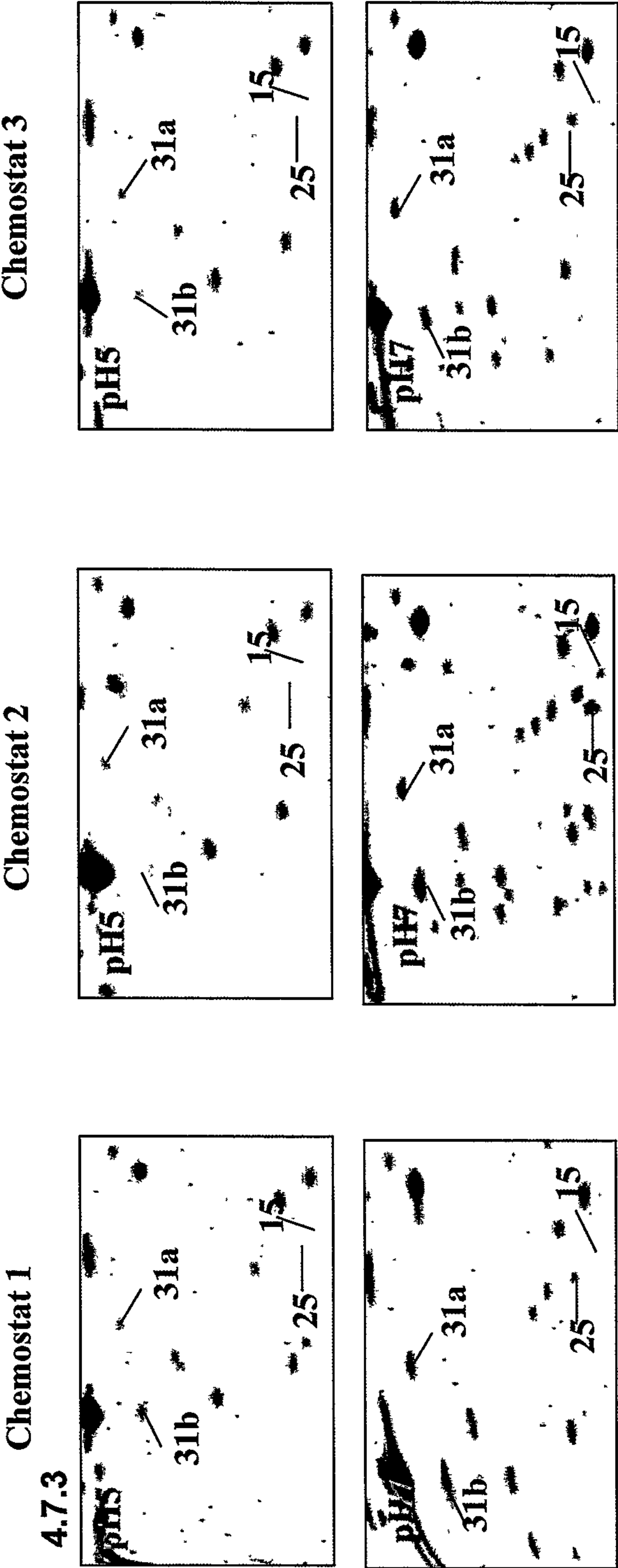
Chemostat 3



4.7.2



4.7C (cont)



Chapter 5- Stress-response proteome of *S. mutans* LT11 during acid tolerant growth.⁸

5.1 Introduction

A key to the survival of *S. mutans* at low pH is its ability to maintain a transmembrane pH gradient (ΔpH), with the interior of the cell more alkaline. This is achieved by upregulation of a proton-translocating F_1F_0 -ATPase that extrudes H^+ as the external environment becomes more acidic. This results in an increased use of ATP for H^+ extrusion and a consequent reduction in cell yield (Belli and Marquis, 1991; Hamilton and Buckley, 1991; Dashper and Reynolds, 1992; Quivey *et al.*, 2001). A series of recent physiological, mutagenic and proteome studies (Chapter 4) (Quivey *et al.*, 1995; Gutierrez *et al.*, 1996; Gutierrez *et al.*, 1999; Jayaraman *et al.*, 1997; Hamilton and Svensäter, 1998; Hahn *et al.*, 1999; Hanna *et al.*, 2001; Kremer *et al.*, 2001; Lemos *et al.*, 2001; Li *et al.*, 2002; Wilkins *et al.*, 2002), however, indicates that *S. mutans* regulates its phenotype in a far more complex fashion than simply increasing its ability to extrude H^+ in response to acid stress. Analysis of this data gave rise to the hypothesis that *S. mutans* redirects carbon from acidic fermentation by-products to more alkaline catabolites. These changes appear to occur in order to minimize the detrimental effects that result from the uncoupling of carbon flux from catabolism, as a consequence of the use of ATP for H^+ extrusion. What remains to be elucidated is the breadth of the stress response in *S. mutans* that allows it to survive and proliferate at low pH.

This chapter reports on such changes when *S. mutans* was grown at steady state in continuous culture.

⁸ Some of the research presented in this chapter has been previously published: Len AC, Harty DWS, Jacques NA. (2004) Stress-responsive proteins are up-regulated in *Streptococcus mutans* during acid tolerance. *Microbiology* **150**: 1339-1351.

5.2 Methods

All methods were as described in Chapter 4.

5.3 Results and Discussion

The increased expression of the proton-translocating F_1F_0 -ATPase that extrudes H^+ from *Streptococcus mutans* (Belli and Marquis, 1991; Hamilton and Buckley, 1991; Dashper and Reynolds, 1992; Quivey *et al.*, 2001) is a key to the survival of the bacterium in an acidic environment. This was reflected in the previously observed increase in the α -(AtpA) and γ -(AtpC) subunits of the F_1 component of the ATPase when the bacterium was grown at pH 5.0 (Chapter 4)(Quivey *et al.*, 2001). However as reported in Chapter 4, a combination of steady-state continuous-culture technology and narrow-range IPG strips, resulted in the resolution of numerous differentially expressed protein spots on Sypro Ruby-stained 2-DGE gels following acid-tolerant growth of *S. mutans* at pH 5.0. Of those identified by MALDI-TOF analysis, 53 protein spots were associated with regulatory and/or stress-responsive pathways (Table 5.1)^{9,10}. These proteins included those involved in DNA replication, transcription, translation, protein folding and proteolysis and are discussed below in light of current knowledge of the possible roles they play in Gram-positive bacteria, particularly oral streptococci.

5.3.1 DNA replication and chromosome integrity

One of the consequences of intracellular acidification is the loss of purines and pyrimidines from DNA, since deoxyribonucleotides are acid labile (Lindahl and Nyberg, 1972). Unrepaired non-instructive DNA damage blocks DNA replication and can be lethal. Aborted replication, however, exposes single-stranded DNA at replication forks and results in the binding of the recombinase protein A, RecA. The DNA-bound RecA protein undergoes a conformational transition to its active form, RecA*. In both *Escherichia coli* and *S. pneumoniae*, RecA* induces an SOS response that can lead to mutation of the DNA or acquisition of pre-evolved functions by

⁹ Table 5.1 has been placed at the end of this Chapter (pp. 151-155)

¹⁰ Triplicate gel spot images have been placed at the end of chapter 5 (Fig. 5.3, 5.4, 5.5 and 5.6; pp. 157-167)

horizontal gene transfer (Taddei *et al.*, 1997; Horst *et al.*, 1999; Steffen *et al.*, 2001; Volkert and Landini, 2001; Bjedov *et al.*, 2003; Katz and Bryant, 2003).

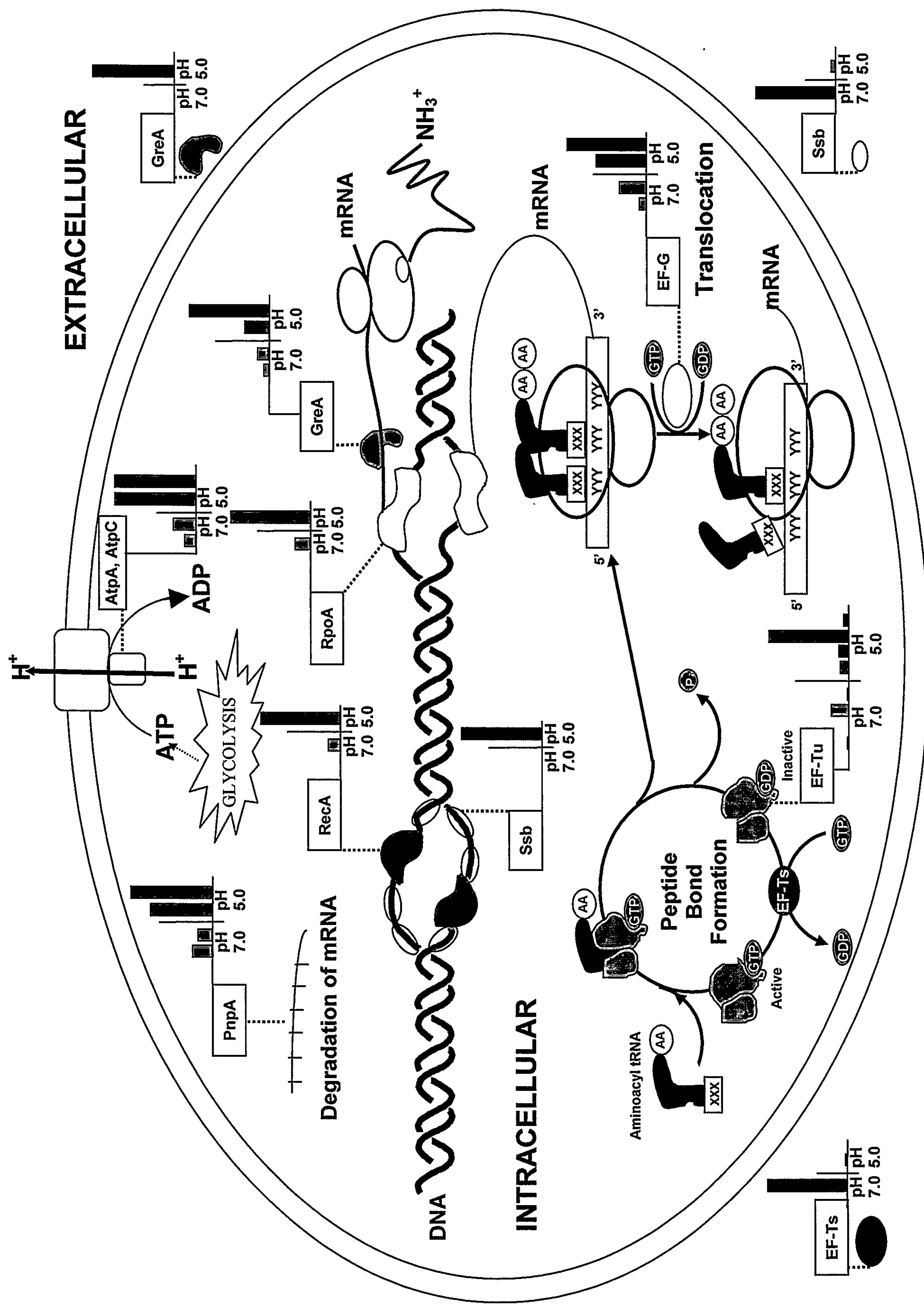
In *S. mutans*, RecA was found to be upregulated 6·8-fold when grown at pH 5·0 (Fig. 5.1, Table 5.1). The physiological effects of low pH on a RecA-deficient strain of *S. mutans* have previously indicated that RecA is required for survival in cells grown at neutral pH when subjected to a rapid drop in pH, but that cells grown at pH 5·0 can diminish the sensitizing effects of RecA deficiency (Quivey *et al.*, 1995). This physiological observation is a consequence of the induction in *S. mutans* during growth at pH 5·0 of an abasic site-specific endonuclease activity which apparently acts independently of the RecA protein (Hahn *et al.*, 1999; Quivey *et al.*, 2001) in a similar manner to an error-prone form of DNA polymerase I (PolA) during the SOS response in *E. coli* (Lackey *et al.*, 1985; Wandt *et al.*, 1997). Even though a DNA polymerase I protein spot was identified in the 2-DGE proteome of *S. mutans*, it represented only the C-terminus of the protein, and was absent at pH 5·0 (Table 5.1).

Along with the enhanced expression of RecA, one of the two single-stranded DNA-binding proteins coded in the *S. mutans* genome, Ssb (Ajdić *et al.*, 2002), was uniquely expressed by growth of *S. mutans* at pH 5·0 (Fig. 5.1, Table 5.1). Both RecA and Ssb are essential for homologous genetic recombination as well as recombinational rescue and DNA repair of chromosomal replication. To the best of our knowledge, the only studies of this protein in streptococci relate to *S. pneumoniae* (Steffen *et al.*, 2002; Katz and Bryant, 2003), making this the first report of a role for Ssb in the acid tolerance of *S. mutans*. From the different roles for recombinational repair and the SOS response currently recognized in Gram-negative and Gram-positive bacteria, and the lack of information available on streptococci, it is clear that further research is needed to understand these processes in the acid-tolerant growth of *S. mutans*.

5.3.2 RNA synthesis and degradation

Under imposed stress conditions, it is self-evident that the DNA repair and protection responses needed for the survival of the cell require the transcription of appropriate genes. While the nature

Figure 5.1. Differentially expressed *S. mutans* proteins involved in replication, transcription and translation from cells grown at pH 7.0 or at pH 5.0. The columns represent the percentage mean DE values for each charged isoform identified on 2-DGE gels, relative to the most highly expressed isoform. Protein spots were either upregulated (green), down-regulated (red), or uniquely expressed (blue), relative to the alternative pH. Truncated forms of proteins are not shown, except for those observed in the extracellular milieu.



of these events has been well documented in *E. coli*, little is known of the mechanism(s) coordinating these events in streptococci, particularly oral streptococci (Volkert and Landini, 2001). Acid tolerant growth in *S. mutans*, however, led to upregulation of the transcription proteins, DNA-directed RNA polymerase α -subunit, RpoA, and two isoforms of the transcription elongation factor (cleavage stimulatory factor) GreA, by 5.4-, 7.5- and 5.1-fold, respectively (Fig. 5.1, Table 5.1). In addition, one isoform of GreA was found to be uniquely expressed in the extracellular milieu at pH 5.0 (Fig. 5.1, Table 5.2)^{11,12}. RNA polymerase forms an elongation complex with its template DNA and the nascent RNA product. While this complex is completely processive, it is responsive both to extrinsic regulatory factors and to intrinsic signals associated with the DNA and RNA that can alter the rate of elongation and lead to a transient pause or arrest of the complex (Nakasone *et al.*, 1998; Erie, 2002). This may be as simple as a lack of nucleotide substrates, a situation that may readily occur in *S. mutans* under acidic conditions, due to the disturbance of anabolic functions (Chapter 4). The main function of GreA is to reactivate RNA polymerase once such a halt has occurred. This is achieved by enhancing the intrinsic cleavage activity of RNA polymerase, thus releasing RNA from the elongation complex, and preventing backward translocation and hydrolysis of the RNA (Fish and Kane, 2002; Opalka *et al.*, 2003). Other than in the current study, elevated levels of GreA have been noted in the Gram-positive bacterium *Staphylococcus aureus*, when challenged with oxacillin (Singh *et al.*, 2001).

Two isoforms of the RNA-degrading enzyme, polynucleotide phosphorylase (polyribonucleotide nucleotidyltransferase [PNPase; PnpA]), were also upregulated by at least 3.1-fold in *S. mutans* in acid-tolerant growth. (Fig. 5.1, Table 5.1). In *E. coli*, two 3'– 5' exonucleases, RNase II and PNPase, are involved in mRNA decay, since the loss of both activities results in the cessation of growth (Donovan and Kushner, 1986). Recent analysis of RNA decay in *E. coli* (Mohanty and Kushner, 2003) has shown that even though RNase II constitutes about 90 % of the exonucleolytic activity in the cell (Deutscher and Reuven, 1991), PNPase plays a greater role in the degradation of mRNA, since it forms part of a multi-protein complex, the 'degradosome', that contains an RhlB RNA helicase capable of removing secondary structure that would impede PNPase activity (Mohanty and Kushner, 2003).

¹¹ Triplicate gel spot images have been placed at the end of chapter 5 (Fig. 5.7; pp 168-170)

¹² Table 5.2 has been placed at the end of this Chapter (pp. 156)

While the level of understanding of RNA decay in Gram-positive bacteria is not as advanced as that of Gram-negative bacteria, *in vitro* RNA decay does not appear to be as severely compromised by the absence of a PNPase activity (Wang and Bechhofer, 1996). In *Bacillus subtilis*, however, a *pnpA* deletion mutant shows pleiotropic effects, including a cold-sensitive-growth phenotype, sensitivity to growth in the presence of tetracycline and multiseptate, filamentous growth (Wang and Bechhofer, 1996). It has been hypothesized that defective processing of specific RNAs in the *pnpA* mutant of *B. subtilis* results in these phenotypes, though there is no direct evidence in support of this contention (Wang and Bechhofer, 1996). To date, no studies of the role of PNPase in oral streptococci appear to have been undertaken, though a 1.9-fold down-regulation of the enzyme has been observed in *Streptococcus oralis* when inoculated and grown in batch culture under aerobic conditions at low pH (Wilkins *et al.*, 2001). Our observation of an increase in PNPase is clearly at odds with this observation. Whether the aerobic conditions used to cultivate *S. oralis* engender an additional oxidative stress that further influences PNPase expression requires investigation.

5.3.3 Translation

The incorporation of correctly encoded amino acids into proteins depends on the attachment of each amino acid to an appropriate tRNA by aminoacyl tRNA synthases (Cooper, 2000). Acid tolerance in *S. mutans* resulted in the upregulation of phenylalanyl- (PheS), alanyl- (AlaS) and two isoforms of threonyl- (ThrS) tRNA synthases, as well as subunits A (GatA) and B (GatB) of glutamyl-tRNA amidotransferase, which is required for the transamidation of misacylated Glu-tRNA^{Gln} to form Gln-tRNA^{Gln} in all Gram-positive bacteria ([Curnow *et al.*, 1997; Harpel *et al.*, 2002]; Table 5.1). In contrast, arginyl-tRNA synthase (ArgS) was down-regulated 20-fold by growth at pH 5.0 (Table 5.1). The other four aminoacyl tRNA synthases previously identified (Chapter 3) did not show any differential expression at pH 5.0.

In *S. mutans*, the 50S and 30S ribosomal subunits are composed of 51 proteins (Ajdić *et al.*, 2002). Of these, five were identified as being upregulated by growth at pH 5.0, four of which existed in more than one charged isogenic form and possessed an observed M_r higher than that predicted from their gene sequence (Table 5.1). While 16 ribosomal proteins have previously

been mapped on 2-DGE gels (Chapter 3), the inability to detect all 51 proteins is most likely due to their low M_r and/or very basic pI (>10.5), which place them at the limit of the resolving power of current 2-DGE technology. Our data imply that the number of ribosomes may increase by a factor of four in a low-pH environment – a suggestion that will require independent confirmation. Protein S1 and the ribosomal protein L10 were also found in the culture medium, but only at significant levels at pH 7.0 (Table 5.2).

In translation, three elongation factors, EF-Tu, EF-Ts and EF-G, are responsible for escorting aminoacyl tRNAs to the ribosome and for translocation of the ribosome along the mRNA (Fig. 5.1; [Cooper, 2000]). A fourfold increase in the expression of the four charged isogenic forms of EF-Tu was observed by growing *S. mutans* at pH 5.0. In each case, the observed M_r of the protein was greater than that predicted from the gene sequence (Table 5.1). Two charged isogenic forms of EF-G were also upregulated at pH 5.0. Most notable, however, was the finding that the reduced amounts of multiple truncated forms of EF-Tu and a C-terminal fragment of EF-G in cells grown at pH 5.0 could be measured at all, as this meant that each protein spot had the same 2-DGE coordinates, irrespective of the growth pH (Table 5.1). This suggested that specific cleavage events had occurred, rather than random proteolysis. A similar observation has been made with *Salmonella enterica* serovar Typhimurium, where two specific proteinases are believed to be responsible for forming elongation factor artifacts (Adams *et al.*, 1999). Even if this were the case with *S. mutans*, the data in Table 5.1 indicate that the cumulative level of both EF-Tu and EF-G would be at least threefold higher during acid-tolerant growth at pH 5.0. Interestingly, no change in the third elongation factor, EF-Ts, was observed, except in the extracellular milieu, where its level was substantially upregulated at pH 7.0 (Fig. 5.1, Table 5.2).

The increase in EF-Tu and EF-G during acid-tolerant growth is also of interest from another standpoint, since it has recently been shown that both proteins behave like chaperones towards unfolded and denatured proteins in *E. coli* (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998). EF-Tu, for example, recognizes the same hydrophobic binding motifs in proteins as the chaperone DnaK (see below; Malki *et al.*, 2002). Furthermore, EF-Ts has been shown to act as a folding template in a chaperone-like manner towards its substrate protein, EF-Tu (Krab *et al.*, 2001).

5.3.4 Molecular chaperones and degradation of misfolded proteins

Early pulse–chase experiments demonstrated an increase in DnaK levels during thermal stress in *S. mutans*, thus confirming the existence of a functional heat-shock response-system in this species (Jayaraman and Burne, 1995). The DnaK chaperone machinery prevents the misfolding and aggregation of ribosome-bound polypeptides (Szabo *et al.*, 1994; Rudiger *et al.*, 1997; Bukau and Horwich, 1998; Agashe and Hartl, 2000). In the current study, proteome analysis identified three isoforms of DnaK in *S. mutans*, which were upregulated 4.6-, 10.9- and 5.0-fold, respectively, at pH 5.0 (Fig. 5.2, Table 5.1). This confirmed a similar observation made by Jayaraman *et al.* (1997), who used the same values of *D* and pH to culture *S. mutans* (Jayaraman *et al.*, 1997).

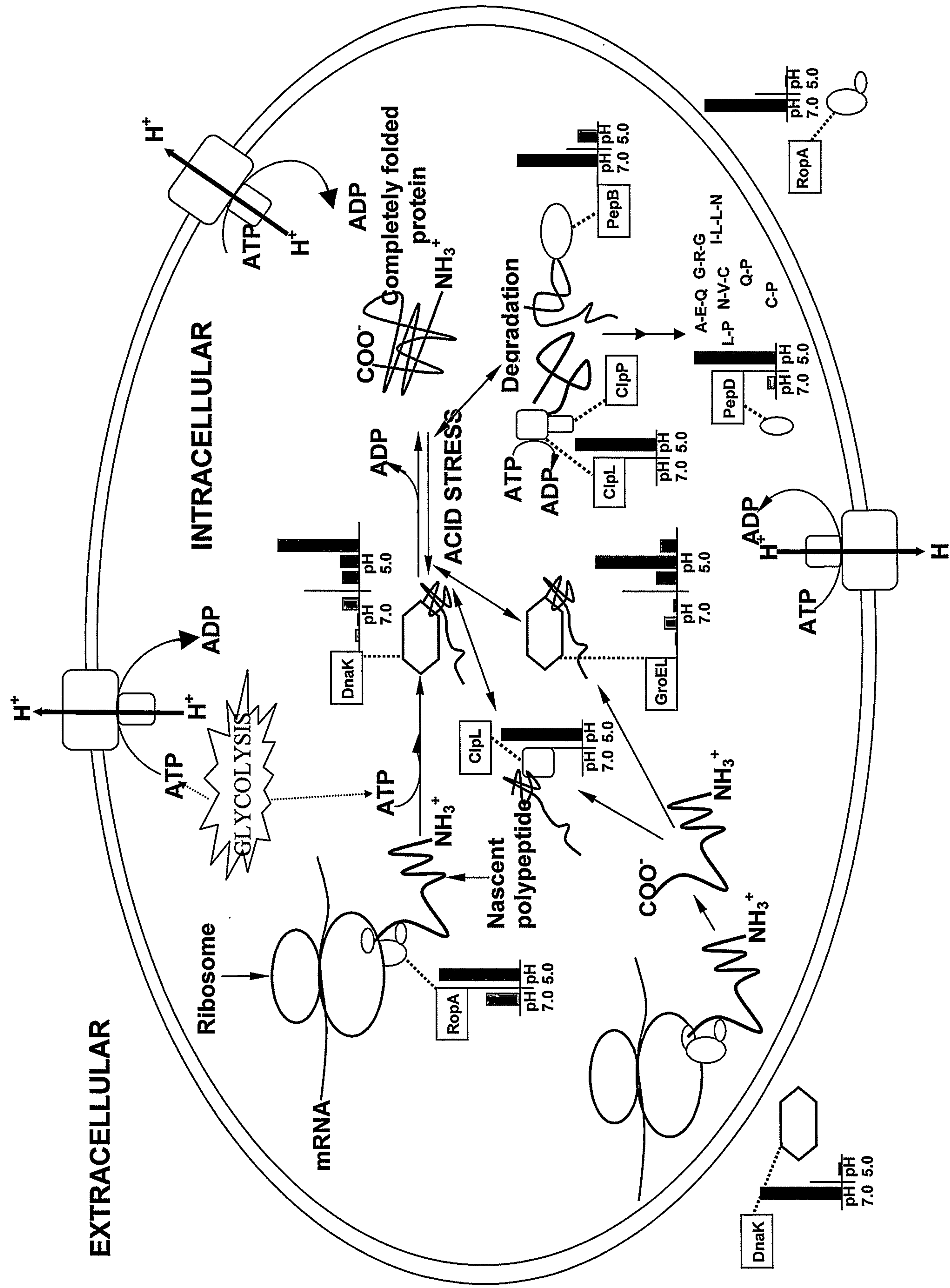
It is now clear that the function of DnaK overlaps with that of another component, trigger factor, RopA (Deuerling *et al.*, 1999; Teter *et al.*, 1999). RopA is a major ATP-independent molecular chaperone with prolyl isomerase activity, which binds to the large ribosomal subunit proteins L23 and L29 near the polypeptide exit site and interacts with nascent polypeptide chains prior to DnaK (Hesterkamp *et al.*, 1996; Kramer *et al.*, 2002).

A 2.4-fold increase in RopA during steady-state growth at pH 5.0 was observed in *S. mutans* (Fig. 5.2, Table 5.1). Other molecular chaperones, such as the bacterial group I chaperonin, GroEL, are indispensable for cell viability (Fayet *et al.*, 1989; Kubota *et al.*, 1995). GroEL is able to capture and refold non-native substrate proteins up to 50–60 kDa, while protecting them from aggregation with other non-native proteins (Braig *et al.*, 1994; Mayhew *et al.*, 1996; Weissman *et al.*, 1996; Xu *et al.*, 1997). Proteome analysis showed the existence of three isoforms of GroEL in *S. mutans*, which were enhanced 20.0-, 6.9- and 7.0-fold, respectively, by growth in an acidic environment (Fig. 5.2, Table 5.1). While similar results have been reported for a 60 kDa chaperonin (most probably GroEL) in aerobic batch cultures of *S. mutans* and *S. oralis* (Wilkins *et al.*, 2001; Wilkins, 2002), the current results differed from those previously reported in continuous culture (Lemos *et al.*, 2001). In the previous study, *S. mutans groEL* mRNA was induced 2.5-fold by an acid shock from pH 7.0 to pH 5.0, but no significant differences in the levels of *groEL* mRNA or GroEL (determined by Western blot analysis) were observed, once steady state was achieved at $D=0.1\text{ h}^{-1}$ (Lemos *et al.*, 2001).

DnaK and RopA were also identified in the *S. mutans* culture fluid. Both proteins were down-regulated, by factors of 34 and 313, respectively, at pH 5.0 (Fig. 5.2, Table 5.2). These levels of DnaK and RopA were equivalent to 39 % and 36 %, respectively, of the steady-state cellular levels at pH 7.0, but were equivalent to less than 0.2 % of the steady-state cellular levels at pH 5.0, implying a loss (or secretion) rate of approximately $4.0\% \text{ h}^{-1}$ of the steady-state cellular levels at pH 7.0, but a negligible rate of loss at pH 5.0 (Jacques *et al.*, 1985). Although previous studies have not considered the extracellular milieu as a source of these proteins (Lemos *et al.*, 2001; Wilkins *et al.*, 2001; Wilkins, 2002), DnaK has recently been identified on the surface of both *S. agalactiae* (Hughes *et al.*, 2002) and *H. influenzae* (Hartmann *et al.*, 2001), and is known to be highly immunogenic in *S. pneumoniae* (Hamel *et al.*, 1997). Whether *S. mutans* has surface-bound molecular chaperones is a matter of conjecture, as our technique of protein extraction did not discriminate between the various cellular compartments. Irrespective of this, the increase in the steady-state cellular levels of DnaK, RopA and GroEL in *S. mutans* at pH 5.0 is consistent with a need for an enhanced complement of molecular chaperones to counteract the denaturing properties of an acidic cytosol when cytosolic pH falls below 6.5 (Dashper and Reynolds, 1992).

Proteins which cannot be folded by molecular chaperones may be targeted for degradation, in order to recycle amino acids for *de novo* protein synthesis (Jenal and Hengge-Aronis, 2003). Among the proteins that can carry out such functions are the ATP-dependent proteases of the Clp family, which possess a dual chaperone/proteinase role. Proteolysis by Clp requires a serine type peptidase, the ClpP subunit, and a regulatory ATPase subunit consisting of several orthologues (Gottesman *et al.*, 1997; Porankiewicz *et al.*, 1999; Lemos and Burne, 2002). In this study of *S. mutans*, only one ATP-binding subunit, ClpL, was expressed, and solely in growth at pH 5.0 (Fig. 5.2, Table 5.1). ClpL homologues appear to be specific to Gram-positive bacteria, as they have not been found in Gram-negative bacteria (Lemos and Burne, 2002; Kwon *et al.*, 2003). To date, information on the role of ClpL is limited to a single study of the heat-shock response in *S. pneumoniae*, in which mutations in the *clpL* and *clpP* genes were found to modulate virulence-gene expression, and purified recombinant ClpL was shown to possess molecular chaperone

Fig. 5.2. Differentially expressed *S. mutans* molecular chaperones and proteinases from cells grown at pH 7.0 or at pH 5.0. The columns represent the percentage mean DE values for each charged isoform identified on 2-DGE gels, relative to the most highly expressed isoform in a given compartment. Protein spots were either upregulated (green), downregulated (red), or uniquely expressed (blue), relative to the alternative pH. Truncated forms of proteins are not shown, except for those observed in the extracellular milieu.



properties (Kwon *et al.*, 2003). Our data suggest that ClpL is upregulated and maintained under low pH conditions, with the implication that it plays a vital role in pH tolerance. It remains to be seen whether ClpL operates solely as a molecular chaperone, or whether it interacts with ClpP to initiate proteolysis.

Two other differentially expressed proteinases were identified by MALDI-TOF, both belonging to the *pep* gene family of proteinases, for which 13 different genes exist in the *S. mutans* genome (<http://www.oralgen.lanl.gov/oralgen/bacteria/smut/>; Ajdić *et al.*, 2002). One of these proteinases, a truncated version of a putative PepB, was down-regulated by a factor of 4.2 in growth at pH 5.0 (Fig. 5.2, Table 5.1). In Group B streptococci, PepB has oligopeptidase activity and has been shown to degrade a variety of small bioactive peptides, as well as the synthetic collagen-like substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala *in vitro* (Lin *et al.*, 1996). The second proteinase was homologous to the dipeptidase PepD of *L. helveticus* (Ajdić *et al.*, 2002). This enzyme does not appear to be similar to the cytoplasmic PepD isolated from *S. mutans* and *S. sanguis* which catalyses the hydrolysis of X-Pro dipeptides (Cowman and Baron, 1997), since the *Lactobacillus* enzyme is not able to hydrolyse di- and tripeptides containing proline (Vesanto E, 1996), PepD was upregulated 12.3-fold at pH 5.0 (Fig. 5.2, Table 5.1).

It is perhaps pertinent to note that proteolysis has another role additional to the recycling of amino acids for *de novo* protein synthesis, and that is to regulate cellular events by degrading regulatory proteins, thereby controlling the response of the cell to an imposed stress. A readjustment in the composition of the cellular proteolytic machinery would therefore most likely have pleiotropic consequences for the cell, as any change in the nature or complement of specific proteinases would be expected to affect proteins that are subject to regulation by proteolytic events (Jenal and Hengge-Aronis, 2003).

5.4 Conclusion

The research described in this chapter made use of the steady-state conditions resulting from

anaerobic continuous culture growth to study alterations in the stress-response proteome of *S. mutans* following adaptation and tolerance to growth at low pH. Using a combination of comparative 2-DGE and PMM analysis, 30 different proteins associated with stress-responsive pathways involved in DNA replication, transcription, translation, protein folding and proteolysis were identified as being associated with acid tolerant growth in *S. mutans*, many of which have previously been the subject of study (Belli and Marquis, 1991; Hamilton and Buckley, 1991; Dashper and Reynolds, 1992; Quivey *et al.*, 2001; Jayaraman *et al.*, 1997; Lemos and Burne, 2002). Among the 25 proteins that were either up-regulated or uniquely expressed were five that have not previously been identified as being associated with acid tolerance in *S. mutans* and/or which have not been studied in any detail in oral streptococci. These were the single-stranded DNA-binding protein, Ssb, the transcription elongation factor, GreA, the RNA exonuclease, polyribonucleotide nucleotidyltransferase (PnpA), and two proteinases, the ATP-binding subunit, ClpL, of the Clp family of proteinases and a proteinase encoded by the *pep* gene family with properties similar to the dipeptidase, PepD, of *Lactobacillus helveticus*. The identification of these and other differentially expressed proteins associated with the acid-tolerant-growth phenotype (Chapter 4) provides new information on targets for mutagenic studies allowing for future assessment of their physiological significance in the survival and proliferation of *S. mutans* in low pH environments.

Table 5.1. Differentially expressed cellular stress-related proteins from *S. mutans* grown at pH 7.0 or pH 5.0.

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI, M _r)		DE Value ^a		Figure ^b
			Theoretical	Observed ^a	pH 7.0	pH 5.0	
ENERGY METABOLISM							
ATPase/Proton motive force							
1	SMu1391	ATP synthase, alpha chain (EC:3.6.3.14) (<i>atpA</i>)	5.0 / 49350	4.87 ± 0.02 / 53310 ± 180	1076 ± 417	8105 ± 229	5.3.2
2	SMu1388	ATP synthase, epsilon chain (EC:3.6.3.14) (<i>atpC</i>)	6.50 / 15882	6.50 / 15880 ^c	1817 ± 567	6468 ± 549	5.3.3
DNA REPLICATION							
DNA replication, restriction, modification, recombination and repair							
3	SMu1892	Recombinase A (<i>recA</i>)	5.0 / 36987	5.40 ± 0.00 / 48280 ± 480	300 ± 157	2040 ± 661	5.4.6
4	SMu0268	DNA-directed DNA polymerase I (EC:2.7.7.7) (<i>polA</i>) ^d	5.3 / 99455	4.70 ± 0.00 / 43110 ± 370	764 ± 334	0	5.3.1
5	SMu1693	Single-strand binding protein (<i>ssb</i>)	5.0 / 18332	4.93 ± 0.03 / 17930 ± 40	0	3773 +/-183	5.4.4
TRANSCRIPTION							
RNA synthesis and modification							
6	SMu1817	DNA-directed RNA polymerase, alpha subunit (EC:2.7.7.6) (<i>rpoA</i>)	3.8 / 34571	4.80 ± 0.00 / 41340 ± 130	1753 ± 472	9428 ± 549	5.3.1
7a	SMu1574	Transcription elongation factor (<i>greA</i>)	4.8 / 17586	4.80 ± 0.00 / 15870 ± 30	2631 ± 495	19600 ± 692	5.3.6
7b	SMu1574	Transcription elongation factor (<i>greA</i>)	4.8 / 17586	4.80 ± 0.00 / 17590 ± 0.0	1137 ± 131	5775 ± 395	5.3.6
Degradation of RNA							
8a	SMu0138	Polyribonucleotide nucleotidyltransferase, alpha chain (EC:2.7.7.8) (<i>pnpA</i>)	5.2 / 80055	5.20 ± 0.00 / 77000 ± 400	371 ± 57	1157 ± 215	5.4.1

Table 5.1 (cont).

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI, M _r)		DE Value ^a		Figure ^b
			Theoretical	Observed ^a	pH 7.0	pH 5.0	
8b	SMu0138	Polyribonucleotide nucleotidyltransferase, alpha chain (EC:2.7.7.8) (<i>pnpA</i>)	5.2 / 80055	5.20 ± 0.00 / 77200 ± 480	261 ± 132	1535 ± 64	5.4.1
TRANSLATION							
Aminoacyl tRNA synthetases							
9a	SMu1441	Threonyl-tRNA synthetase 1 (EC:6.1.1.3) (<i>thrS</i>)	5.4 / 74758	5.33 ± 0.03 / 73280 ± 790	41 ± 41	741 ± 81	5.4.1
9b	SMu1441	Threonyl-tRNA synthetase 1 (EC:6.1.1.3) (<i>thrS</i>)	5.4 / 74758	5.40 ± 0.00 / 74760 ± 0.0	101 ± 101	1236 ± 384	5.4.1
10	SMu0592	Alanyl-tRNA synthetase (EC:6.1.1.7) (<i>alaS</i>)	5.2 / 97162	5.28 ± 0.02 / 88230 ± 600	441 ± 147	1283 ± 363	5.4.1
11	SMu1904	Arginyl-tRNA synthetase (EC:6.1.1.19), (<i>argS</i>)	6.0 / 63574	6.10 ± 0.00 / 58590 +/- 580	1715 ± 84	84 ± 84	5.5.1
12	SMu1374	Phenylalanyl-tRNA synthetase, alpha subunit (EC:6.1.1.20) (<i>pheS</i>)	6.2 / 39296	6.10 ± 0.00 / 40200 ± 1050	137 ± 137	6936 ± 555	5.5.1
13	SMu1657	Glutamyl-tRNA (Gln) amidotransferase, B subunit (EC:2.6.-.-) (<i>gatB</i>)	5.1 / 53501	5.08 ± 0.02 / 50250 ± 280	604 ± 123	4105 ± 127	5.4.2
14	SMu1658	Glutamyl-tRNA (Gln) amidotransferase, A subunit (<i>gatA</i>)	5.2 / 52893	5.18 ± 0.03 / 52960 ± 360	520 ± 41	2233 ± 299	5.4.2
Ribosomal proteins							
15a	SMu1097	30S ribosomal protein S1 (<i>rpsA</i>)	5.0 / 43686	5.00 ± 0.00 / 50170 ± 250	2780 ± 72	13569 ± 233	5.3.2
15b	SMu1097	30S ribosomal protein S1 (<i>rpsA</i>)	5.0 / 43686	4.90 ± 0.00 / 50250 ± 300	865 ± 381	7463 ± 2305	5.3.2
16a	SMu1847	30S ribosomal protein S2 (<i>rpsB</i>)	5.0 / 29093	5.00 ± 0.00 / 33150 ± 110	412 ± 75	2240 ± 333	5.4.3
16b	SMu1847	30S ribosomal protein S2 (<i>rpsB</i>)	5.0 / 29093	5.10 ± 0.00 / 33050 ± 50	1772 ± 88	8036 ± 644	5.4.3

Table 5.1 (cont).

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI, M _r)		DE Value ^a		Figure ^b
			Theoretical	Observed ^a	pH 7.0	pH 5.0	
17a	SMu1836	30S ribosomal protein S3 (<i>rpsC</i>)	10.0 / 24445	10.02 ± 0.20 / 27300 ± 2900	1593 ± 68	6411 ± 233	5.6.1
17b	SMu1836	30S ribosomal protein S3 (<i>rpsC</i>)	10.0 / 24445	10.00 ± 0.36 / 27610 ± 1780	241 ± 121	3990 ± 424	5.6.1
18a	SMu1480	50S ribosomal protein L1 (<i>rplA</i>)	9.4 / 26674	9.35 ± 0.26 / 29770 ± 2070	1415 ± 256	5528 ± 591	5.6.1
18b	SMu1480	50S ribosomal protein L1 (<i>rplA</i>)	9.4 / 26674	9.55 ± 0.15 / 28190 ± 1850	2188 ± 91	7812 ± 833	5.6.1
18c	SMu1480	50S ribosomal protein L1 (<i>rplA</i>)	9.4 / 26674	9.25 ± 0.03 / 30050 ± 2040	483 ± 173	2003 ± 247	5.6.1
19	SMu0872	50S Ribosomal protein L7/L12 (<i>rplL</i>)	4.5 / 12413	4.48 ± 0.02 / 9580 ± 130	8173 ± 5016	76144 ± 7464	5.3.4
<i>Translation elongation factors</i>							
20a	SMu0324	Elongation factor G (EF-G) (<i>fusA</i>)	4.8 / 76664	4.80 ± 0.00 / 76660 ± 0.0	1141 ± 389	8076 ± 1212	5.3.2
20b	SMu0324	Elongation factor G (EF-G) (<i>fusA</i>)	4.8 / 76664	4.80 ± 0.00 / 76510 ± 0.0	4402 ± 1045	12935 ± 2204	5.3.2
20c	SMu0324	Elongation factor G (EF-G), (<i>fusA</i>) ^d	4.8 / 76664	5.00 ± 0.00 / 25170 ± 40	925 ± 92	45 ± 17	5.4.4
21a	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9 / 43919	4.88 ± 0.04 / 56820 ± 0.0	1460 ± 204	10156 ± 972	5.3.2
21b	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9 / 43919	4.88 ± 0.04 / 56520 ± 0.0	0	11115 ± 1751	5.3.2
21c	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9 / 43919	4.91 ± 0.04 / 57000 ^c	20729 ± 10281	98340 ± 4379	5.3.2
21d	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9 / 43919	4.98 ± 0.04 / 57410 ± 440	1145 ± 707	5303 ± 1400	5.3.2
21e	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9 / 43919	4.98 ± 0.02 / 52630 ± 320	5189 ± 415	820 ± 169	5.3.5
21f	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^d	4.9 / 43919	4.80 ± 0.00 / 26640 ± 200	3827 ± 48	1825 ± 161	5.3.4

Table 5.1 (cont).

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI, M _r)		DE Value ^c		Figure ^b
			Theoretical	Observed ^c	pH 7.0	pH 5.0	
21g	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^d	4.9 / 43919	5.00 ± 0.00 / 22340 ± 40	3017 ± 172	618 ± 73	5.3.4
21h	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^d	4.9 / 43919	5.10 ± 0.02 / 20300 ± 70	2280 ± 204	572 ± 76	5.4.4
21i	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^d	4.9 / 43919	5.36 ± 0.31 / 20360 ± 234	2625 ± 331	476 ± 55	5.4.2
21j	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^e	4.9 / 43919	4.45 ± 0.05 / 13720 ± 170	4353 ± 331	830 ± 52	5.4.4
21k	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^e	4.9 / 43919	4.54 ± 0.05 / 12960 ± 130	1531 ± 49	57 ± 67	5.4.4
21l	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^e	4.9 / 43919	5.00 ± 0.00 / 25170 ± 330	3868 ± 296	1240 ± 177	5.4.5
21m	SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>) ^e	4.9 / 43919	5.30 ± 0.00 / 22260 ± 190	3247 ± 508	1099 ± 76	5.4.5
PROTEIN CONFORMATION							
Molecular Chaperones							
22a	SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6 / 65286	4.60 ± 0.00 / 64840 ± 620	1204 ± 172	5529 ± 1165	5.3.3
22b	SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6 / 65286	4.60 ± 0.00 / 65180 ± 90	576 ± 299	6277 ± 1936	5.3.3
22c	SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6 / 65286	4.60 ± 0.00 / 65290 ± 0.0	5673 ± 248	28513 ± 3337	5.3.3
23a	SMu1774	60 kDa chaperonin (<i>groEL</i>)	4.7 / 57101	4.70 ± 0.00 / 58090 ± 240	535 ± 168	10692 ± 844	5.3.1
23b	SMu1774	60 kDa chaperonin (<i>groEL</i>)	4.7 / 57101	4.70 ± 0.00 / 57380 ± 220	6405 ± 344	44202 ± 4753	5.3.1

Table 5.1 (cont).

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI, M _r)		DE Value ^a		Figure ^b
			Theoretical	Observed ^a	pH 7.0	pH 5.0	
23c	SMu1774	60 kDa chaperonin (<i>groEL</i>)	4.7 / 57101	4.70 ± 0.00 / 57100 ± 110	1217 ± 251	8576 ± 2025	5.3.1
24	SMu0082	Peptidyl-prolyl isomerase (trigger factor; <i>ropA</i>)	4.5 / 47486	4.51 ± 0.02 / 54560 ± 400	6653 ± 623	16183 ± 1705	5.3.3
Degradation of proteins and peptides							
25	SMu0587	Oligoendopeptidase B (EC:3.4.24.15) (<i>pepB</i>)	5.0 / 69724	4.80 ± 0.00 / 43090 ± 0.0	5517 ± 585	1324 ± 113	5.3.1
26	SMu0868	ATP-dependent Clp protease, ATP-binding subunit (EC:3.4.21.-) (<i>ClpL</i>)	5.0 / 77186	4.98 ± 0.05 / 71010 ± 50	0	3492 ± 379	5.3.2
27	SMu1190	Dipeptidase (EC:3.4.13.18) (<i>pepD</i>)	5.0 / 53037	5.06 ± 0.03 / 52120 ± 310	135 ± 9	1665 ± 191	5.4.2

^a Mean ± SEM (*n* = 3).

^b Figure containing the corresponding protein spot.

^c Part of the complement of random landmark proteins used to align gels and which were used for the calculation of the observed pI and M_r in accordance with the requirement of the z3 software.

^d C-terminus of protein.

^e N-terminus of protein.

Table 5.2. Differentially expressed extracellular stress-related proteins from *S. mutans* grown at pH 7.0 or pH 5.0.

Spot No	Gene ID	Identification (gene)	2-DGE Co-ordinates (pI, M _r)		DE Value ^a		Figure
			Theoretical	Observed ^a	pH 7.0	pH 5.0	
DNA REPLICATION							
DNA replication, restriction, modification, recombination and repair							
28	SMu1693	Single-strand binding protein (<i>ssb</i>)	5.0 / 18332	4.93 ± 0.03 / 17930 ± 40	0.0	3773 +/-183	5.7.3
TRANSCRIPTION							
RNA synthesis and modification							
29	SMu1574	Transcription elongation factor (<i>greA</i>)	4.8 / 17586	4.80 ± 0.00 / 17590 ± 0.0	1137 ± 131	5775 ± 395	5.7.3
TRANSLATION							
Translation elongation factors							
30	SMu1846	Elongation factor Ts (<i>eflS</i>)	4.9 / 37719	4.92 ± 0.04 / 41650 ± 530	1594 ± 644	12 ± 10	5.7.2
Ribosomal proteins							
31	SMu1097	30S ribosomal protein S1 (<i>rpsA</i>)	5.0 / 43686	4.88 ± 0.06 / 47100 ± 500	1162 ± 196	0.0	5.7.2
32a	SMu1480	50S ribosomal protein L10 (<i>rpIJ</i>)	4.9 / 17667	4.70 ± 0.00 / 14960 ± 100	930 ± 1030	11 ± 1	5.7.3
32b	SMu1480	50S ribosomal protein L10 (<i>rpIJ</i>)	4.9 / 17667	4.80 ± 0.00 / 10300 ± 90	2137 ± 273	81 ± 1	5.7.3
CELLULAR PROCESSES							
Molecular Chaperones							
33	SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6 / 65286	4.60 ± 0.00 / 65290 ± 0	2882 ± 739	85 ± 6	5.7.1
34	SMu0082	Peptidyl-prolyl isomerase (trigger factor; <i>ropA</i>)	4.5 / 47486	4.47 ± 0.02 / 56130 ± 340	1787 ± 98	6 ± 6	5.7.2

^aMean ± SEM (n = 3).

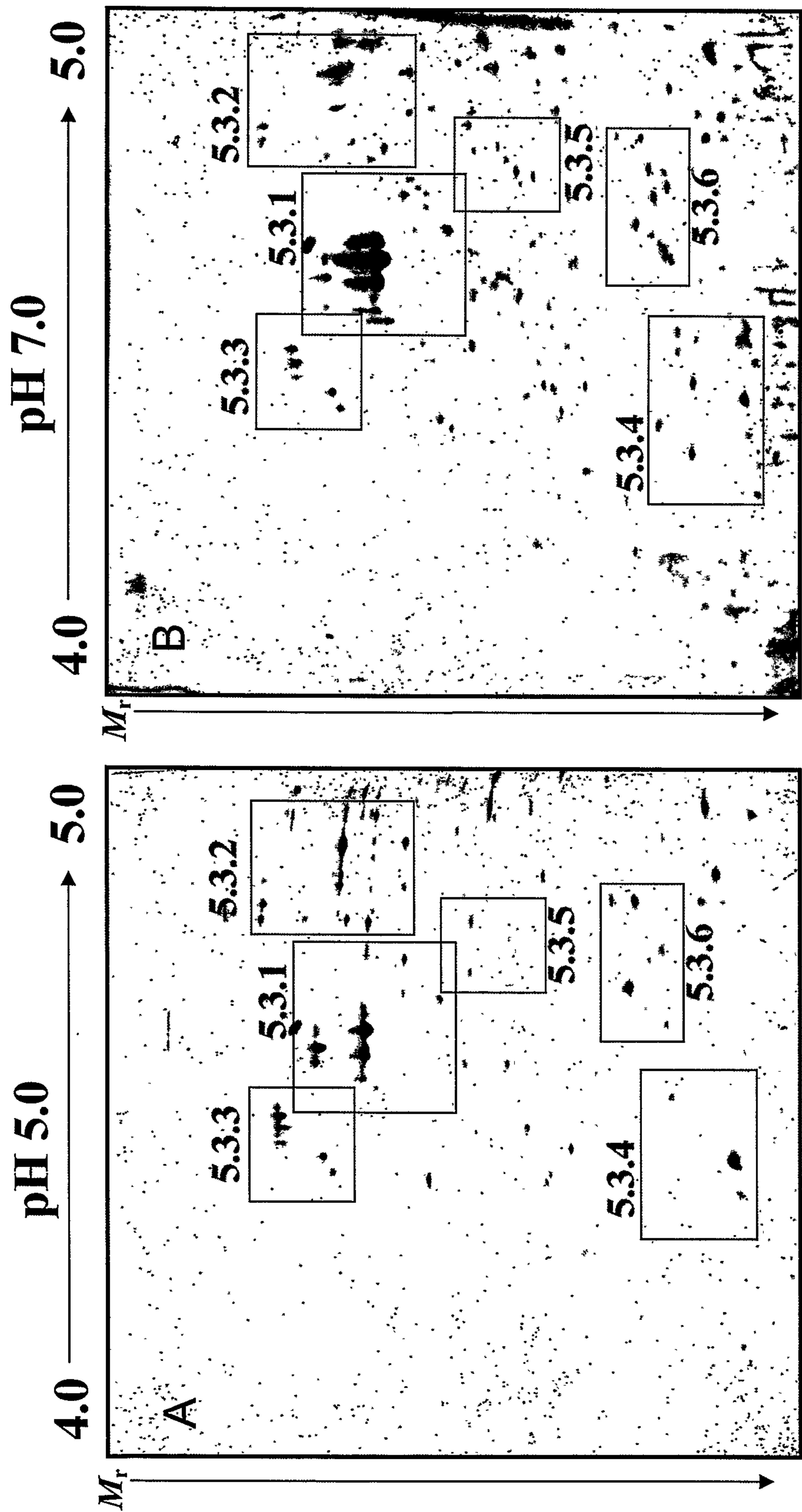


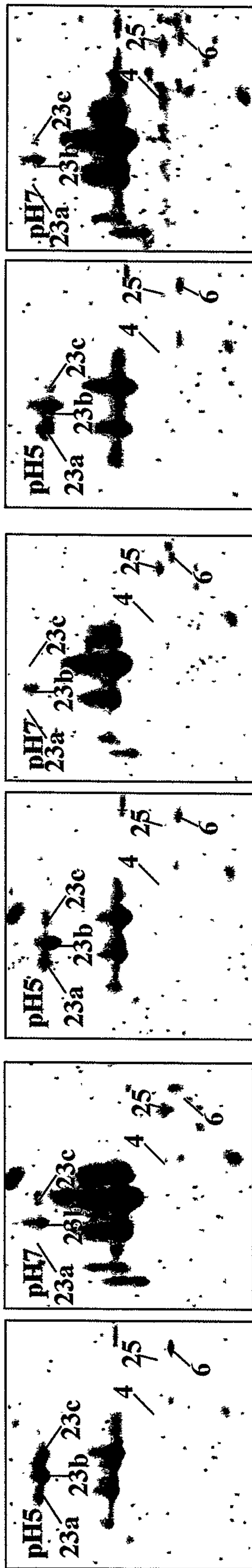
Figure 5.3. 2-DGE Sub-proteome maps (pH 4.0 – 5.0) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas (5.3.1, 5.3.2, 5.3.3, 5.3.4, 5.3.5 and 5.3.6) depict spots that are differentially expressed. Enlarged triplicates of boxed areas, corresponding to their respective chemostat 2-DGE gels are illustrated in 5.3C with spot numbers corresponding to those in Table 5.1.

530

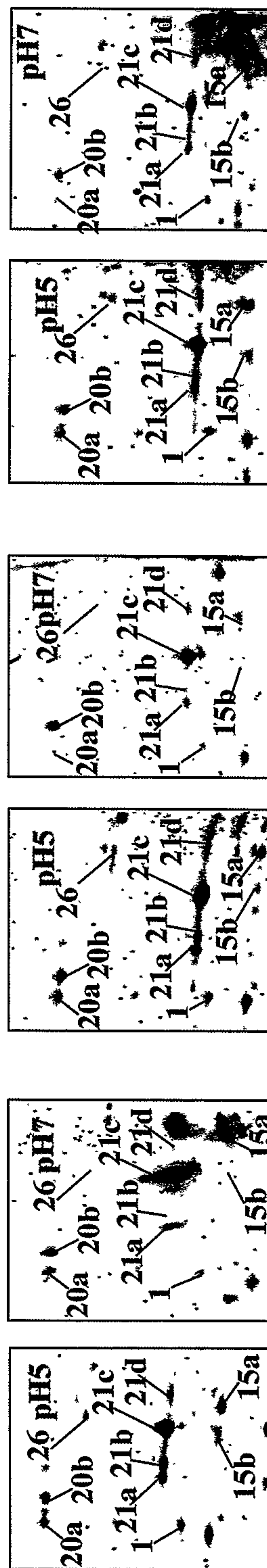
5.3.1 Chemostat 1

Chemostat 2

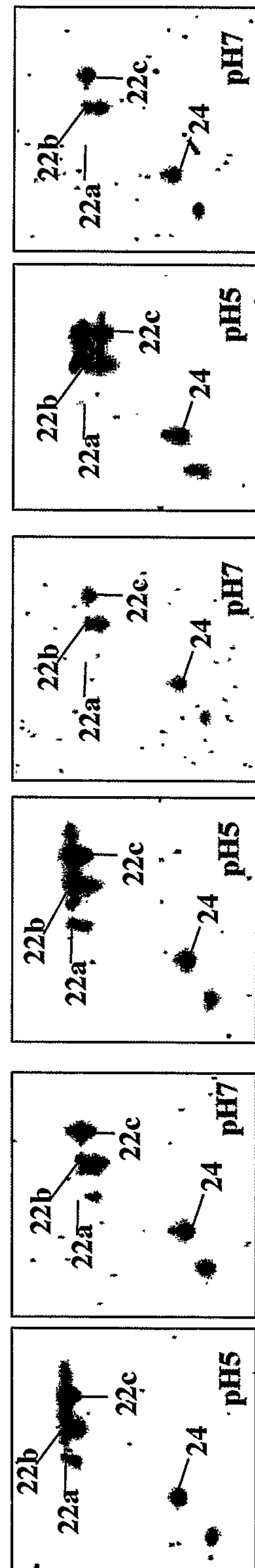
Chemostat 3



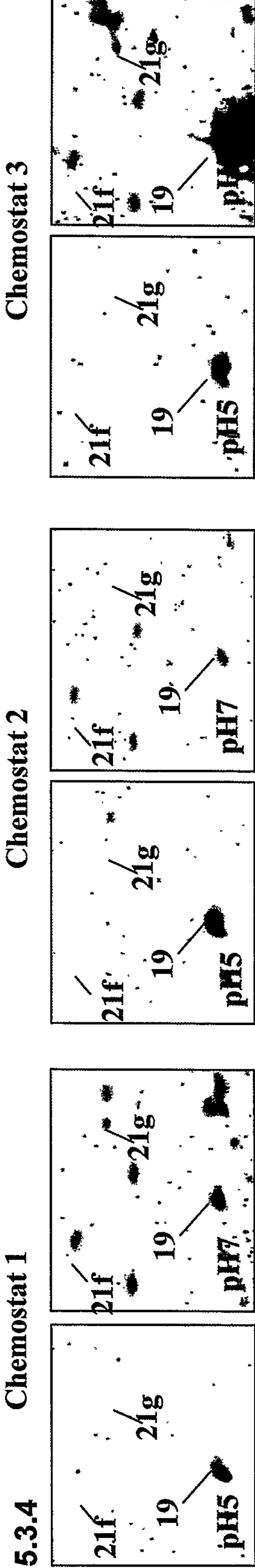
5.3.2



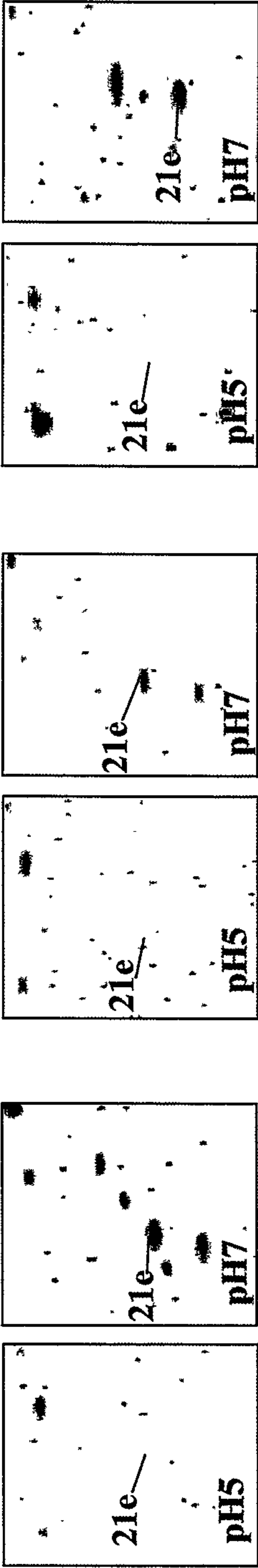
5.3.3



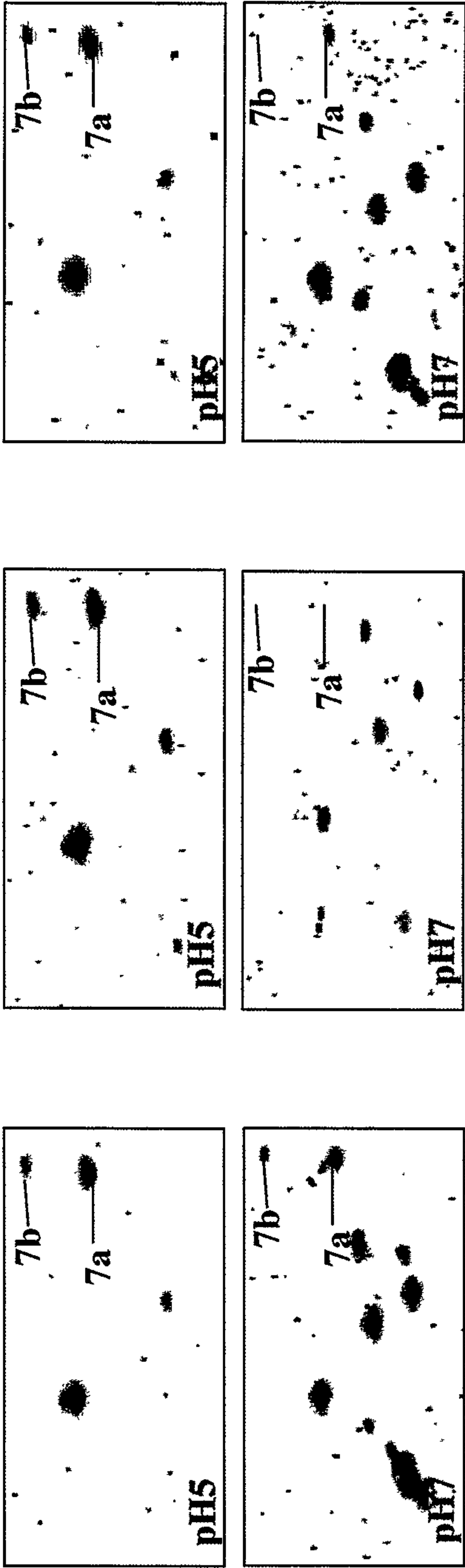
5.3C (cont)



5.3.5



5.3.6



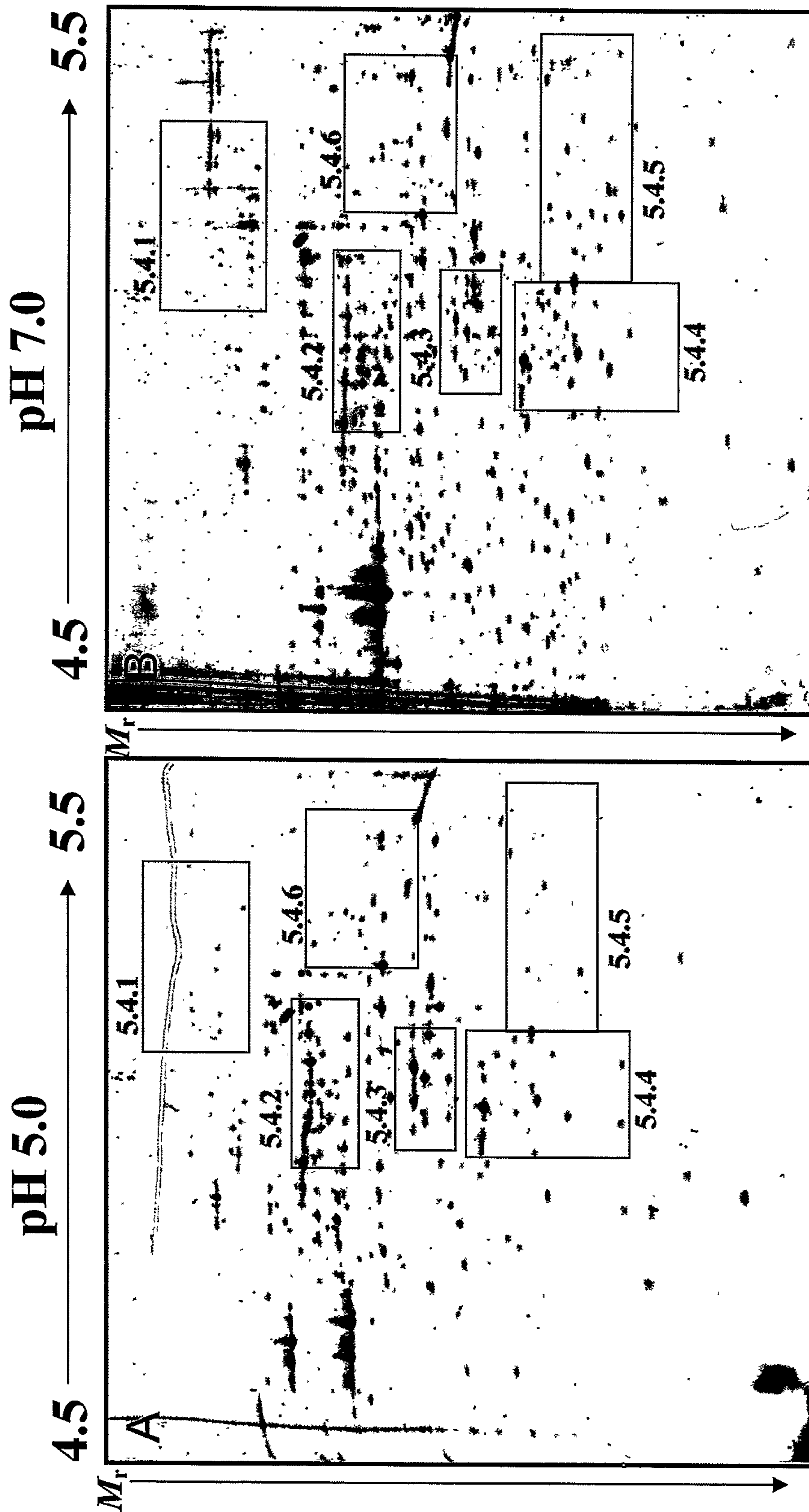
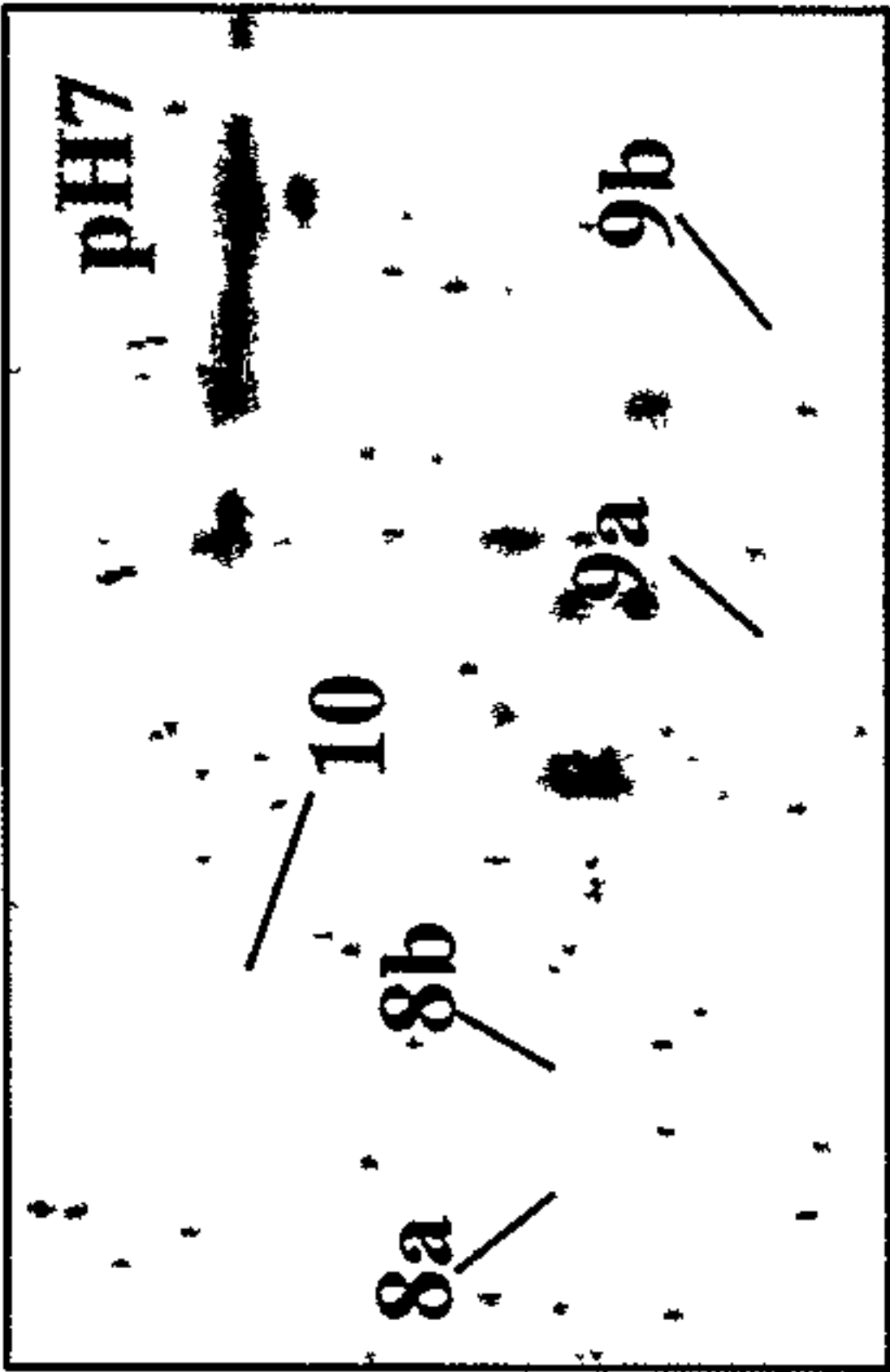
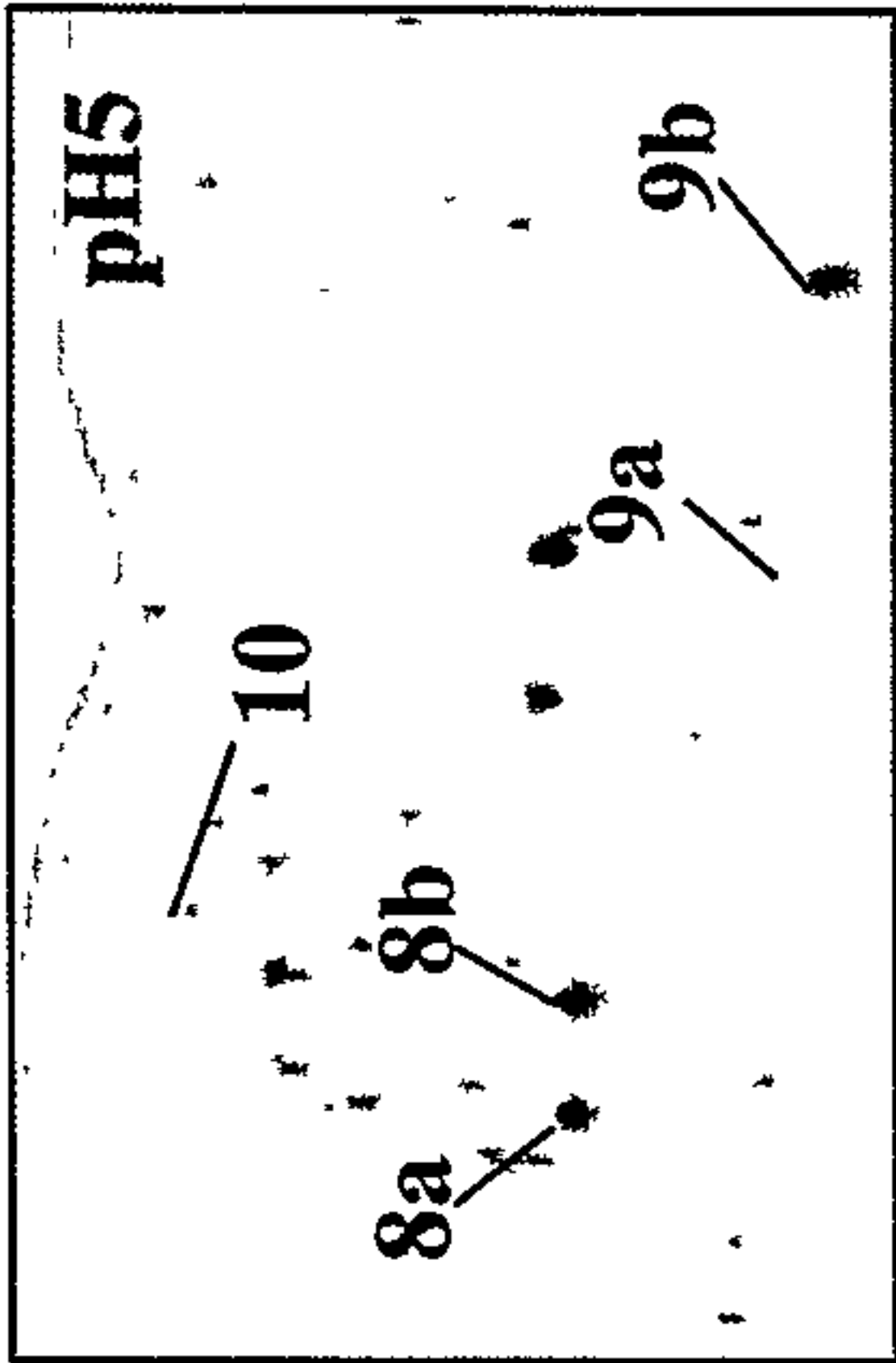


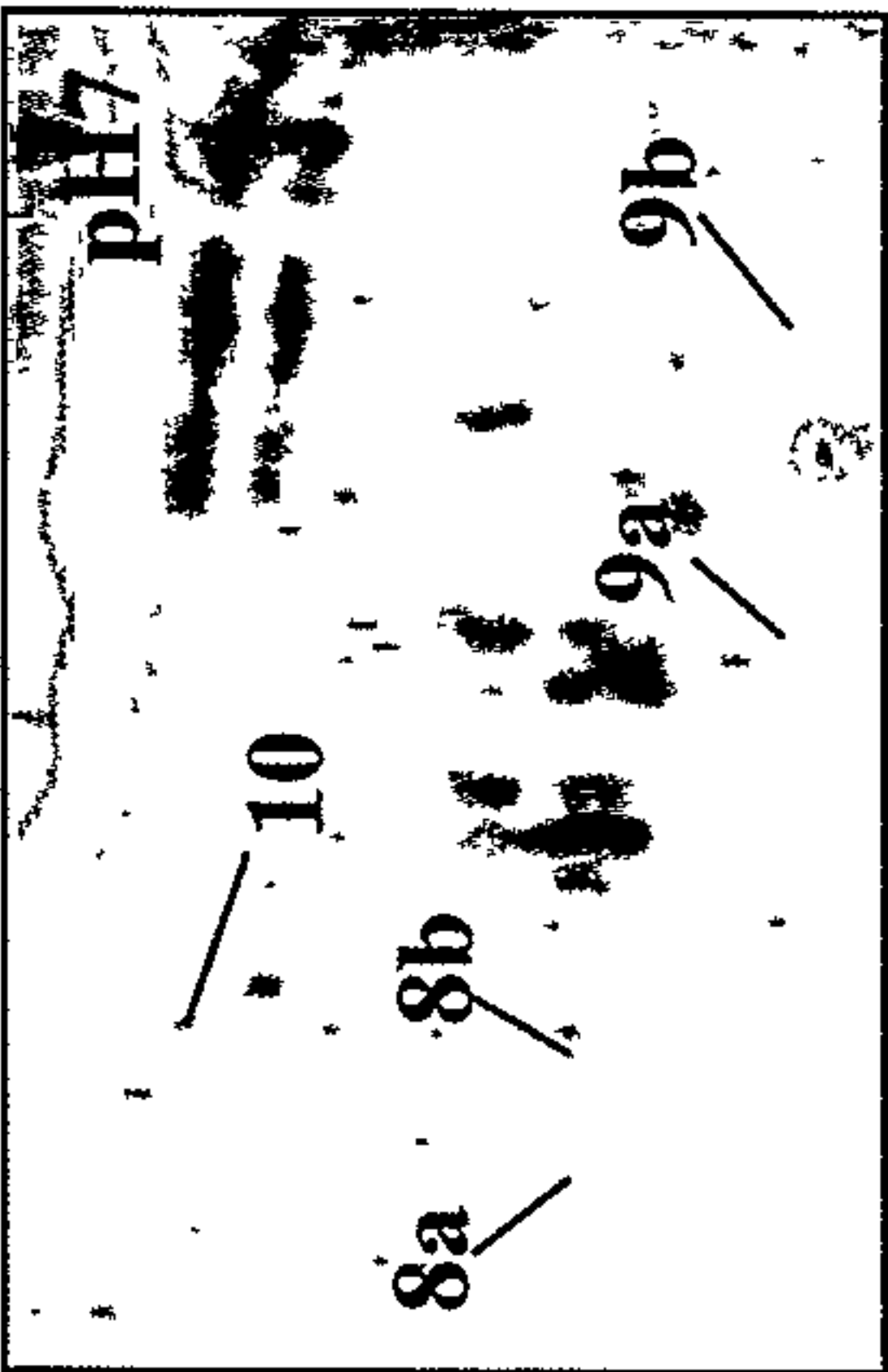
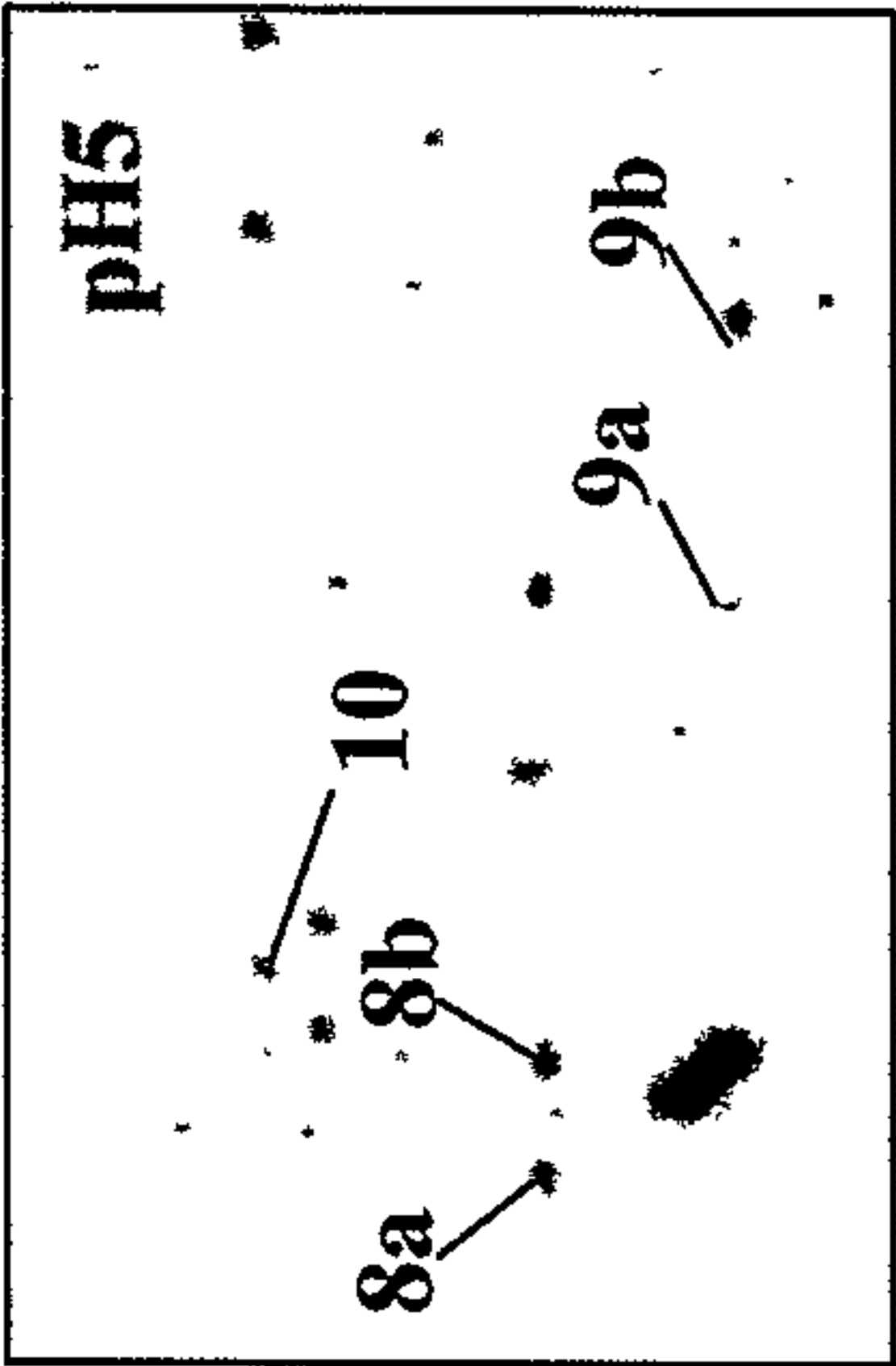
Figure 5.4. 2-DGE Sub-proteome maps (pH 4.5 – 5.5) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas (5.4.1, 5.4.2, 5.4.3, 5.4.4, 5.4.5, 5.4.6) depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed areas, corresponding to their respective chemostat 2-DGE's, are illustrated in 5.4C with spot numbers corresponding to those in Table 5.1.

5.4C

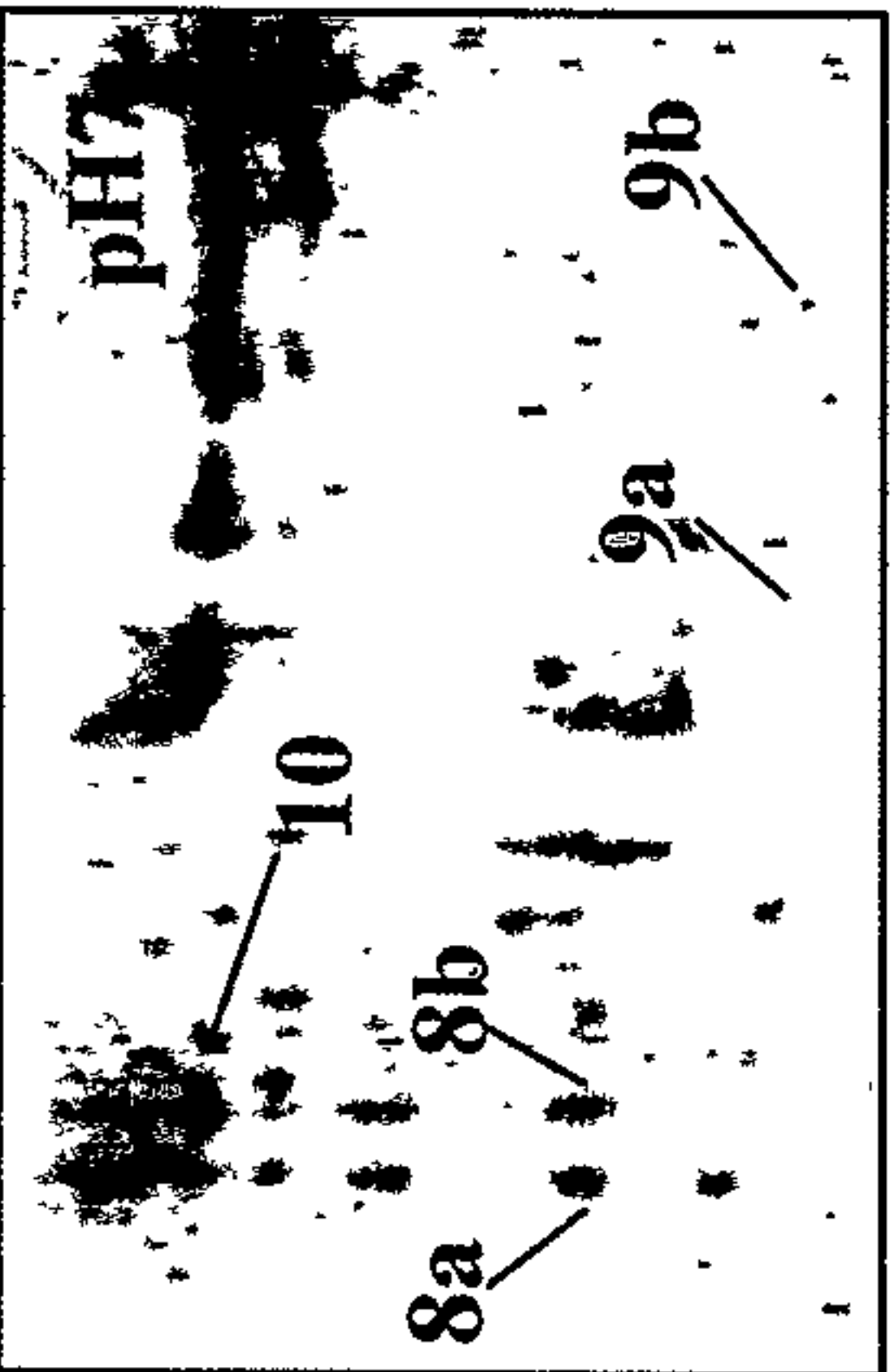
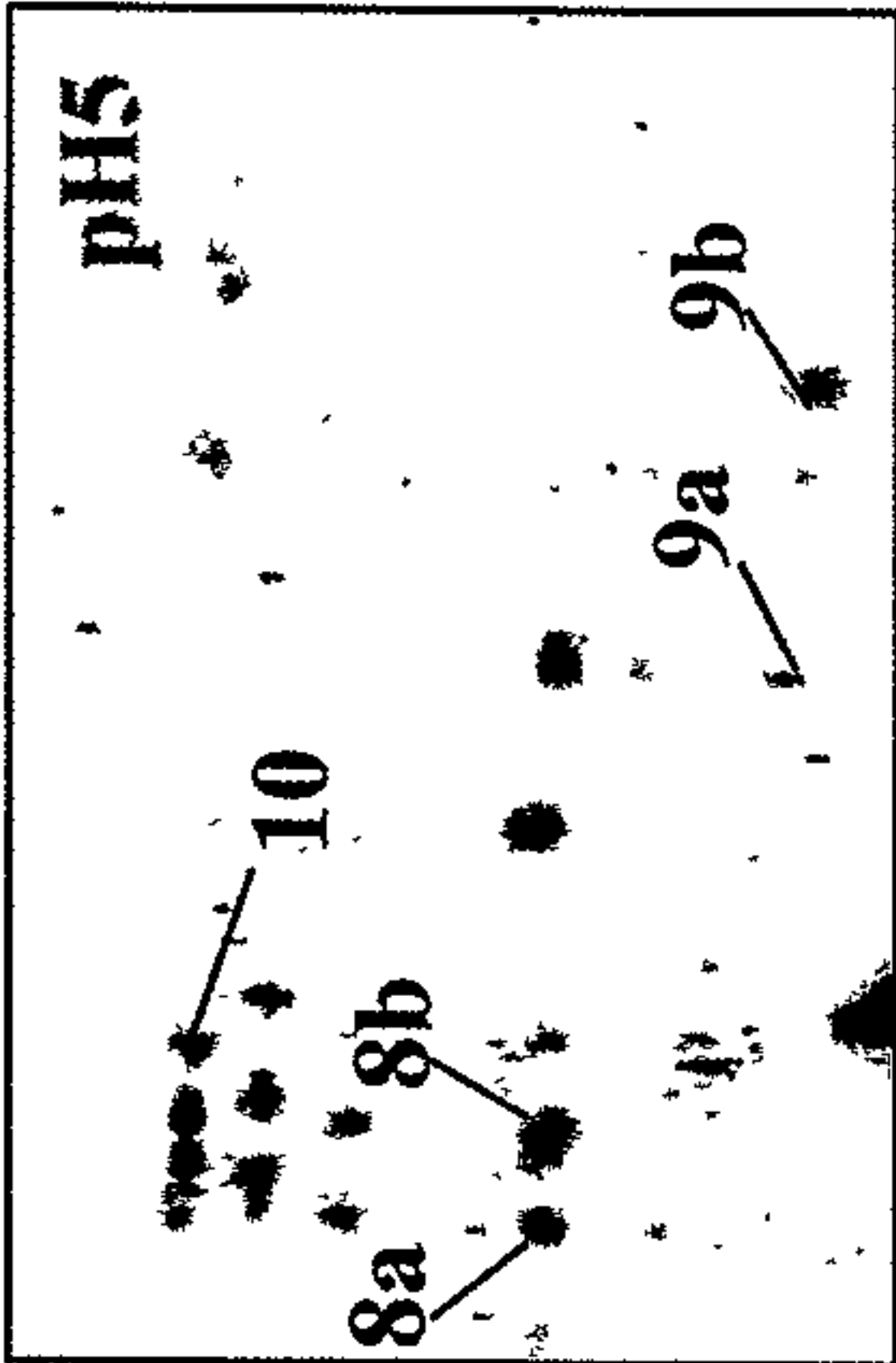
5.4.1 Chemostat 1



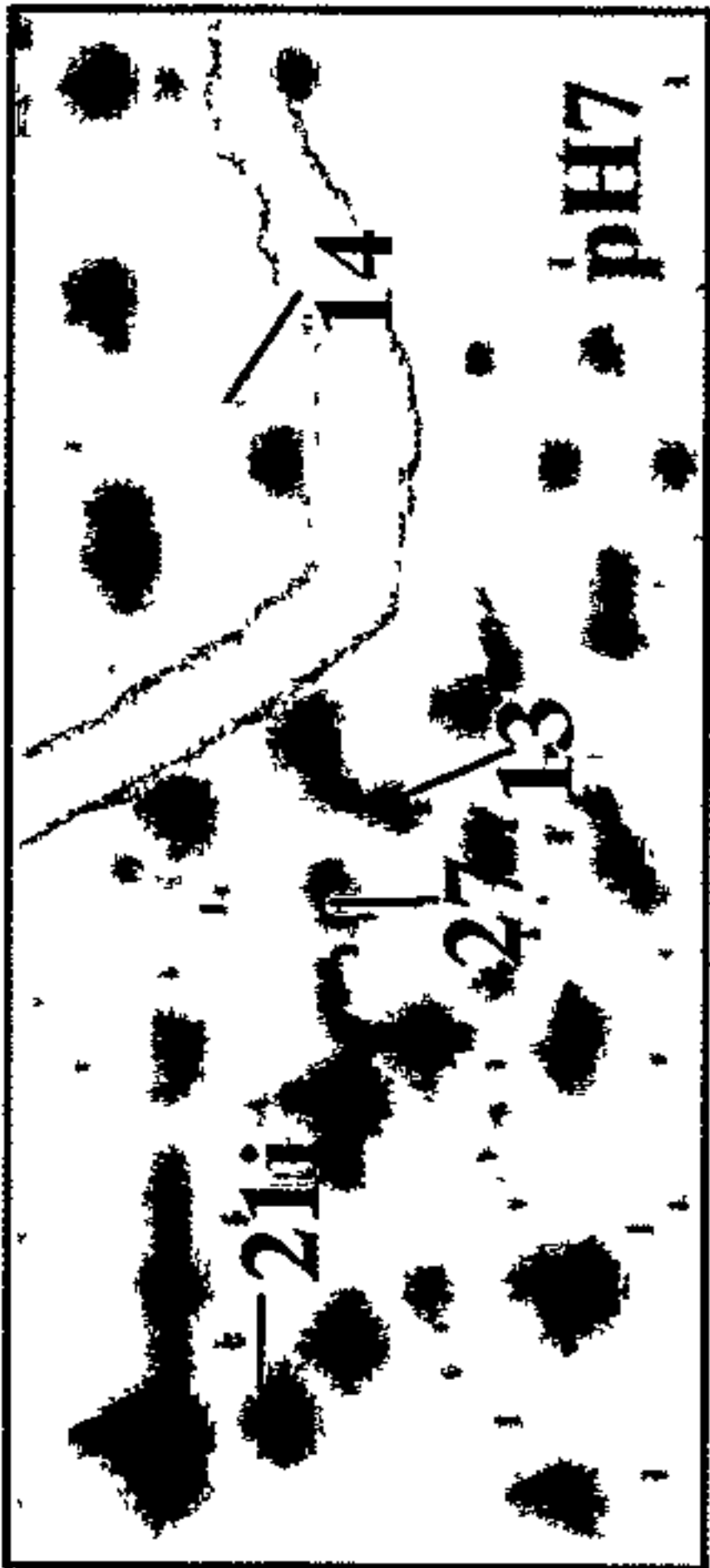
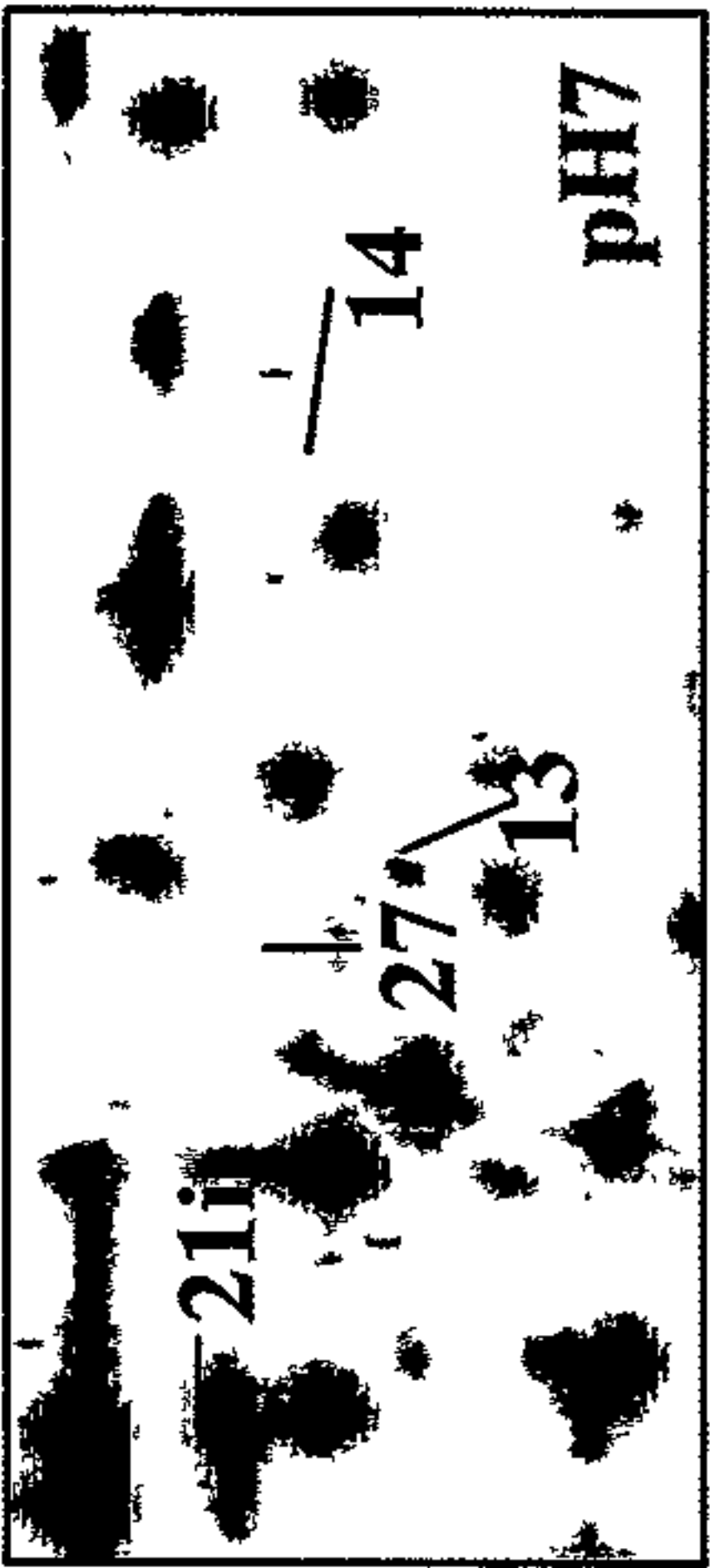
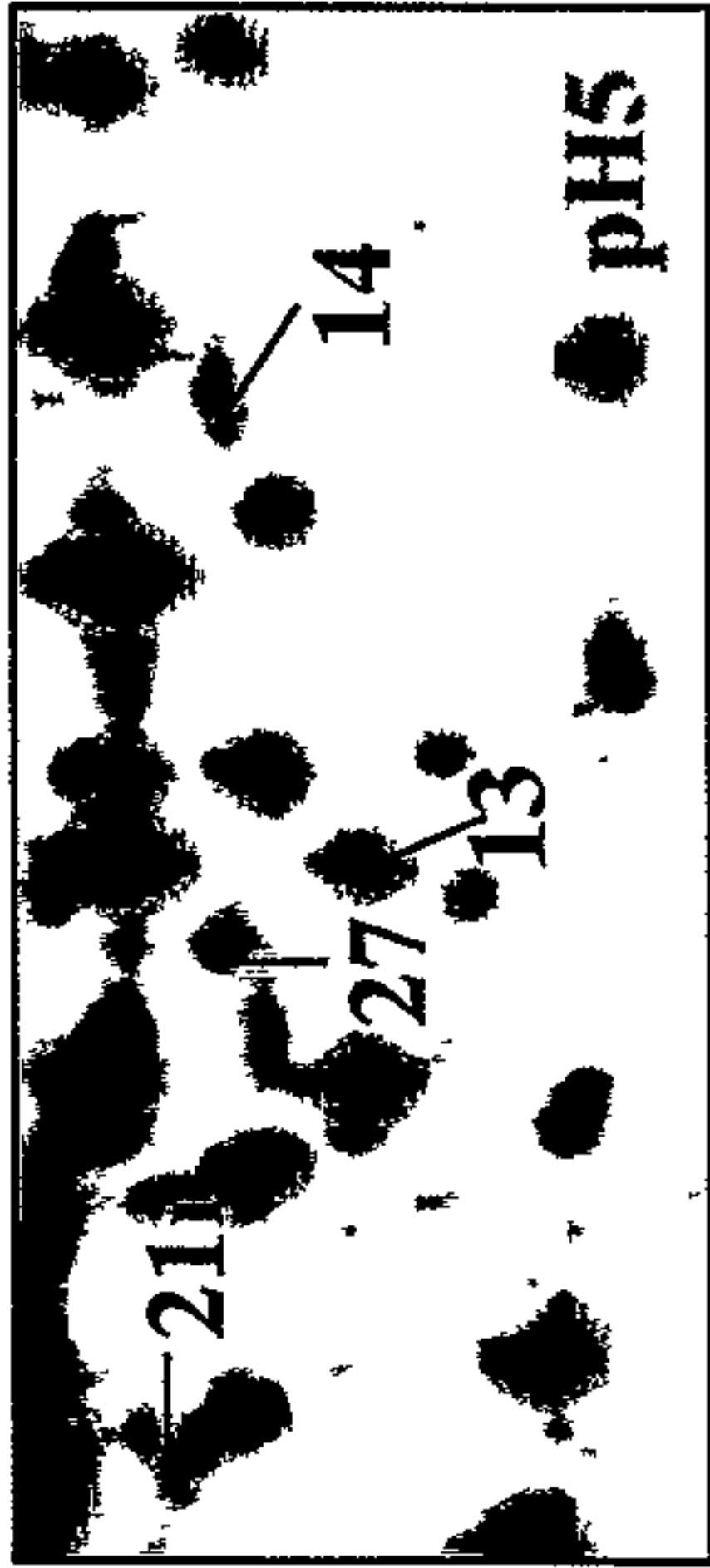
Chemostat 2



Chemostat 3



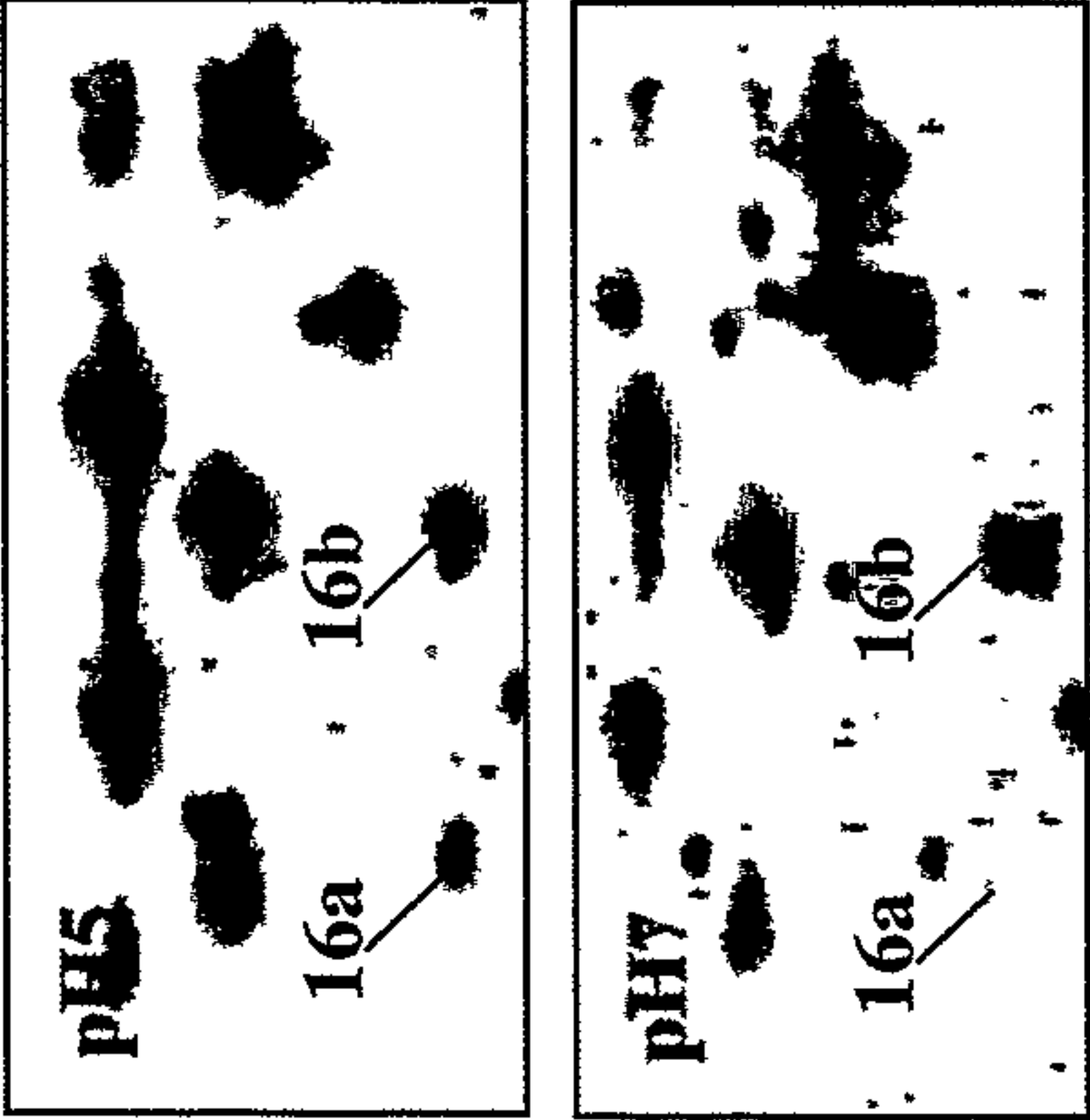
5.4.2



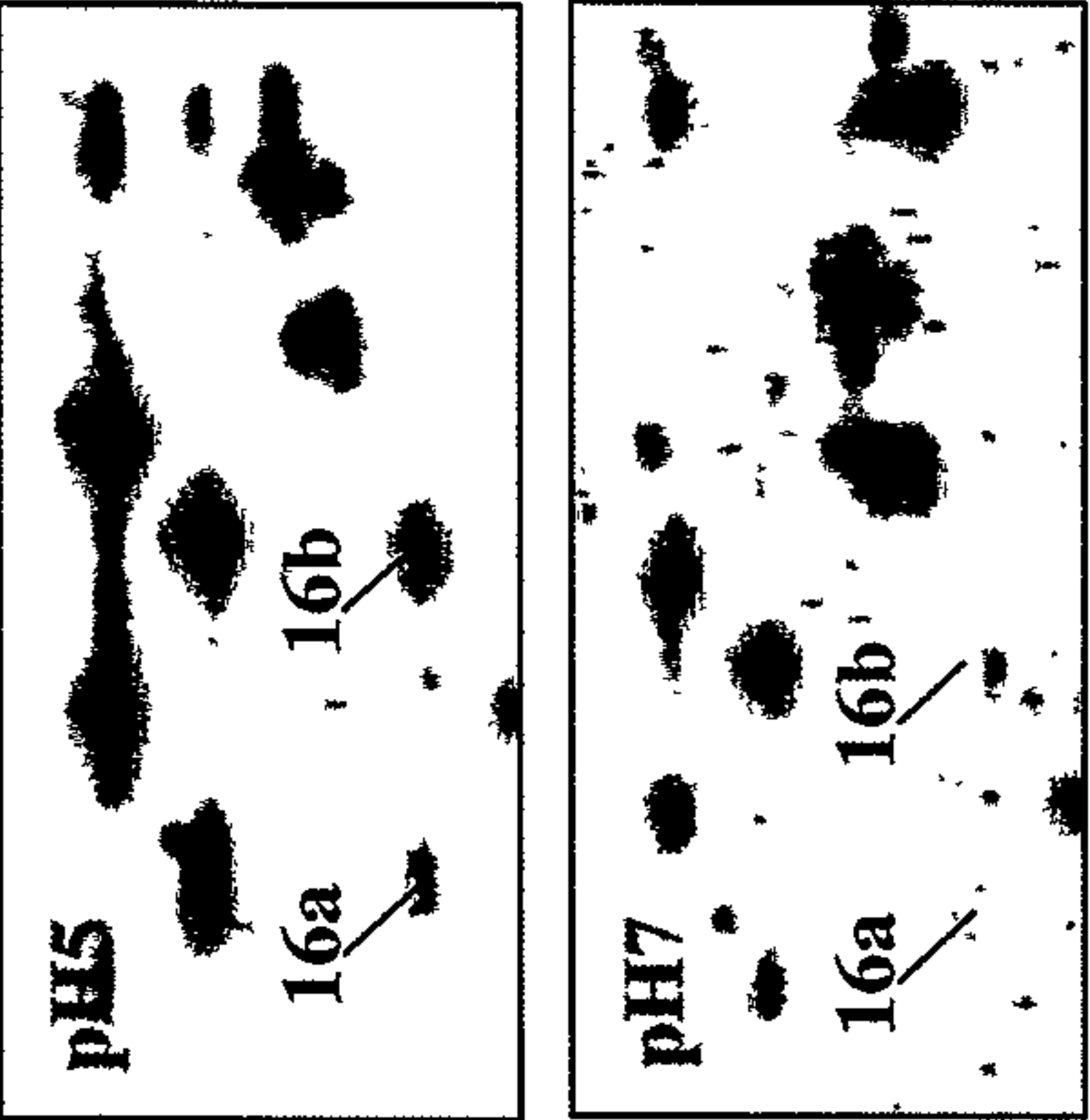
5.4C (cont)

Chemostat 1

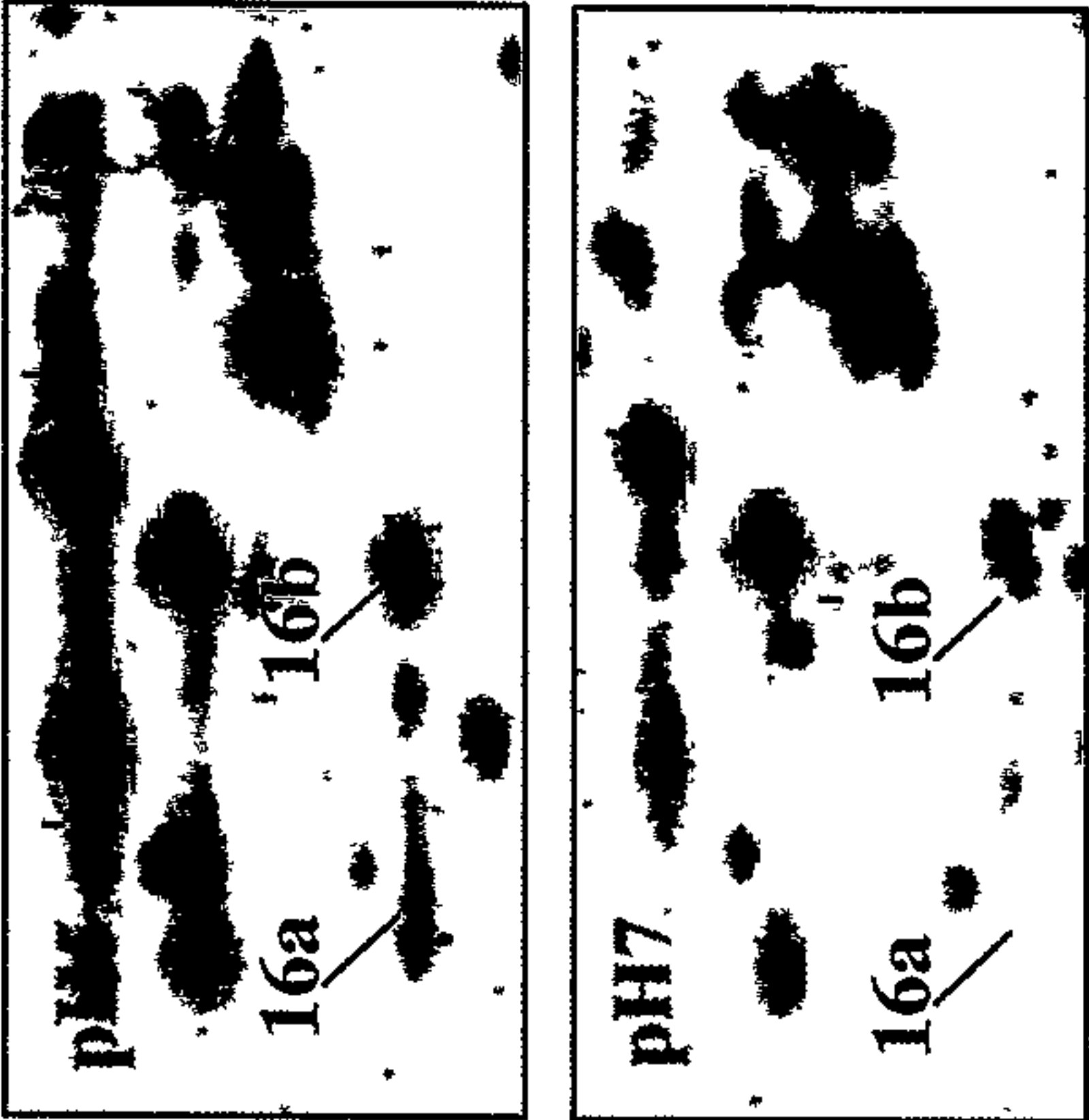
5.4.3



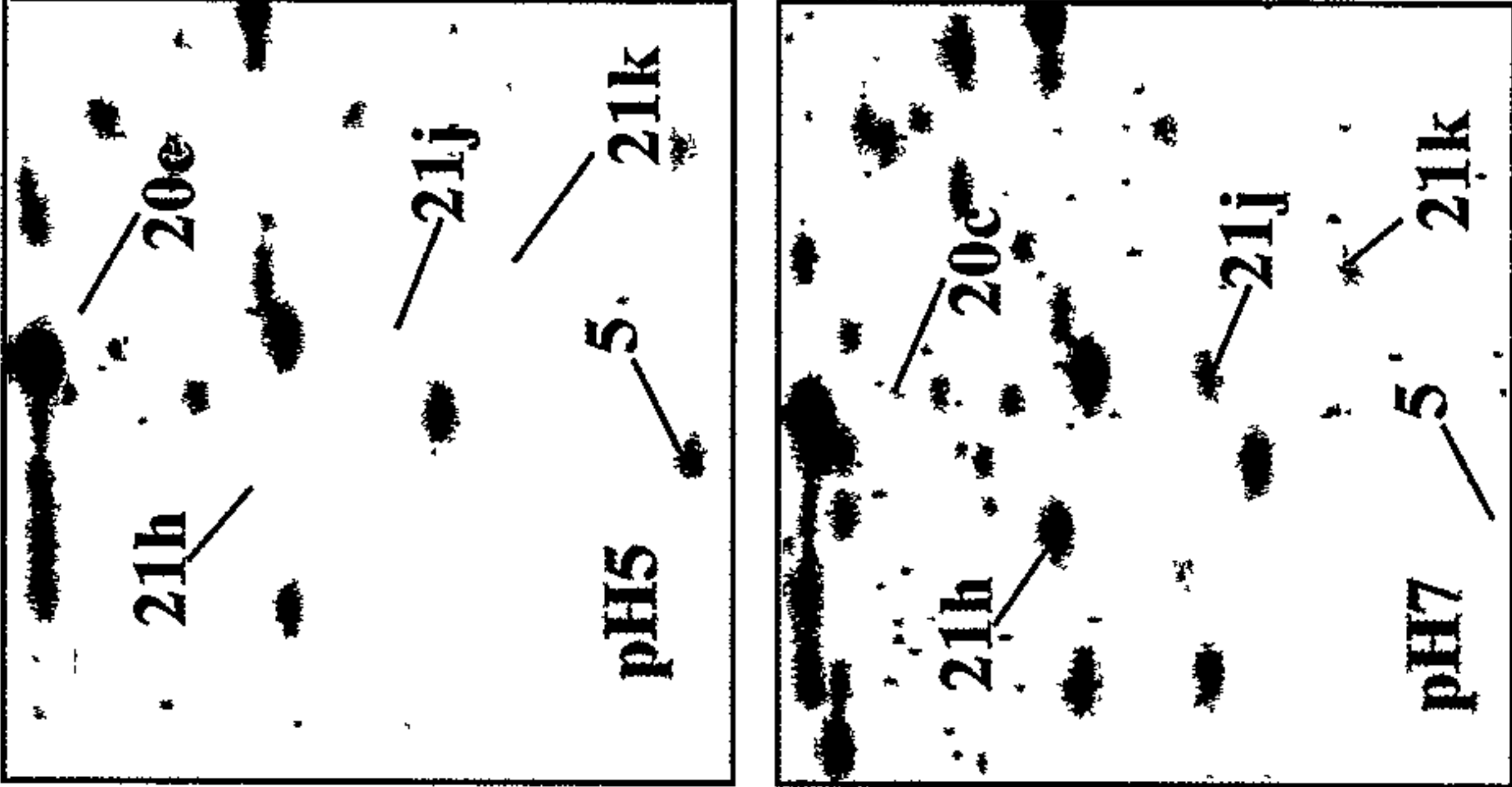
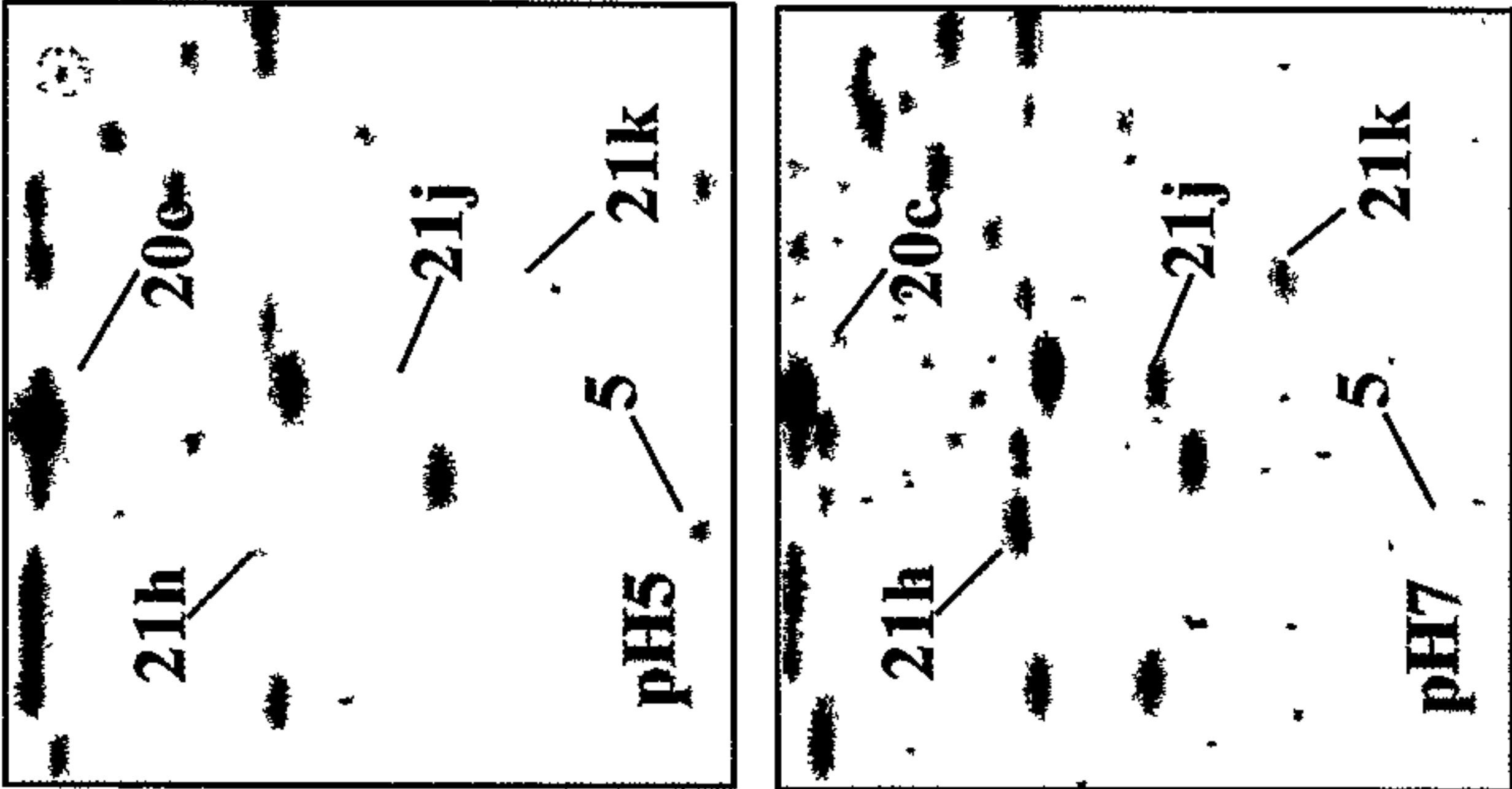
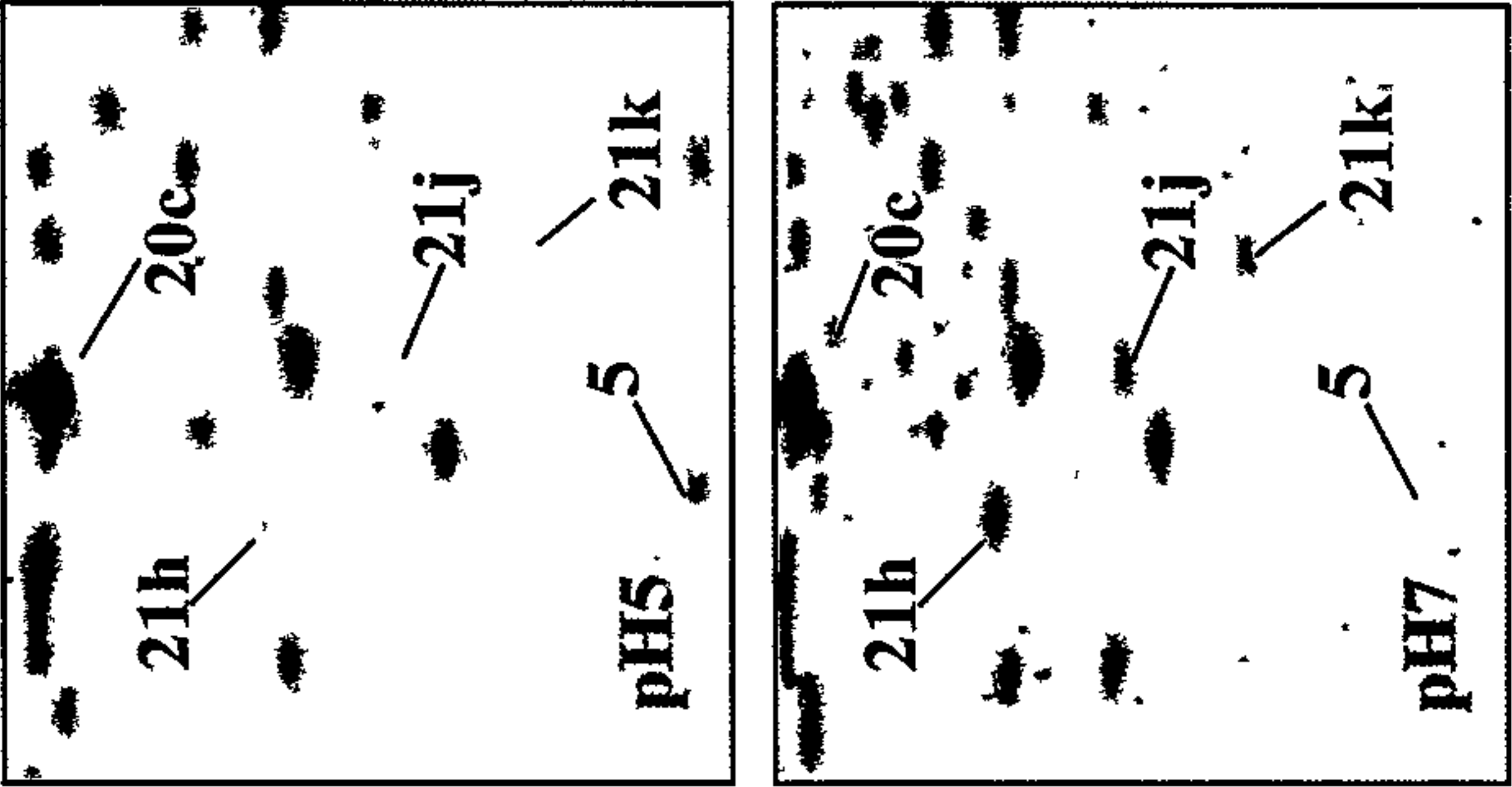
Chemostat 2



Chemostat 3

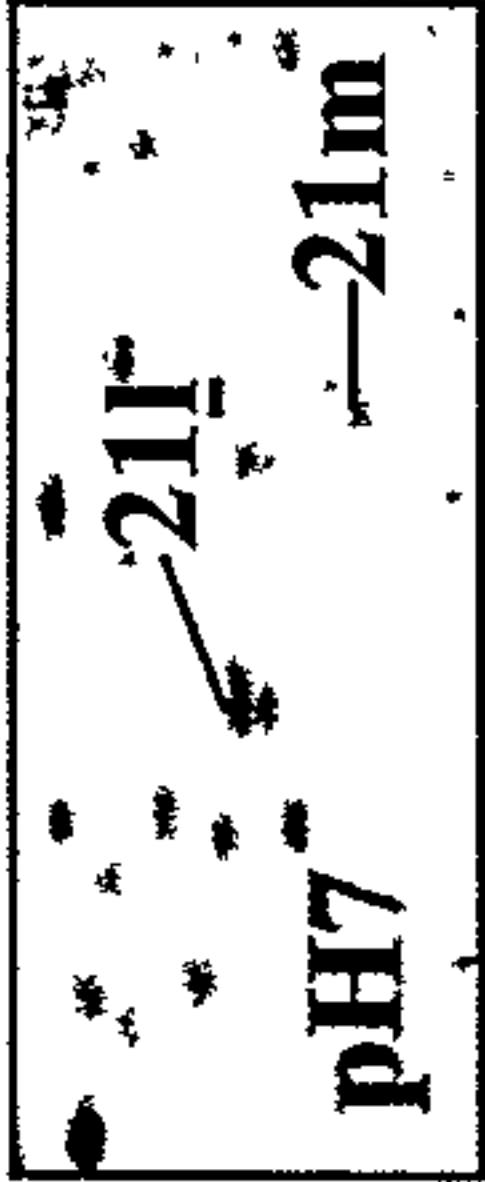
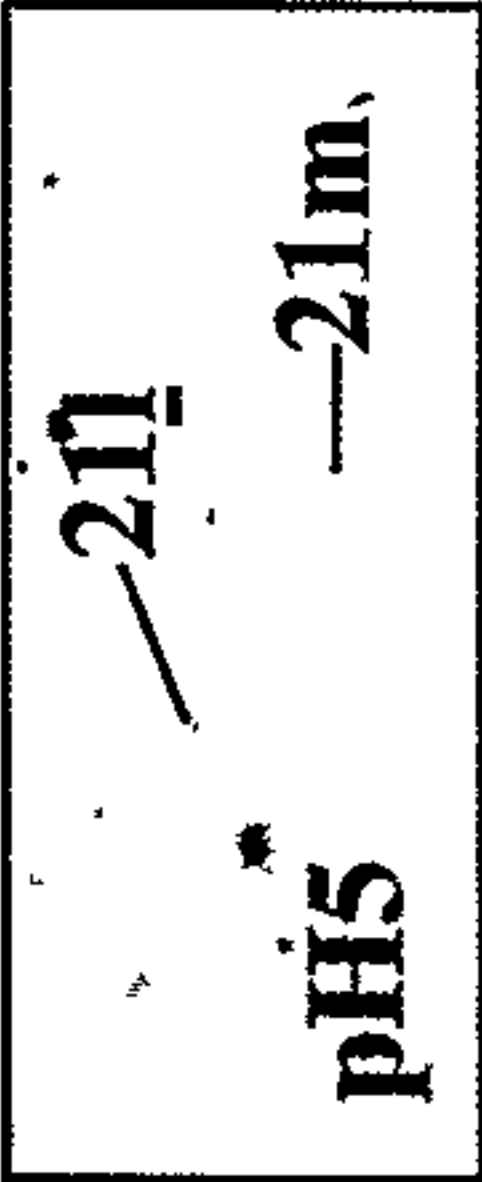


5.4.4

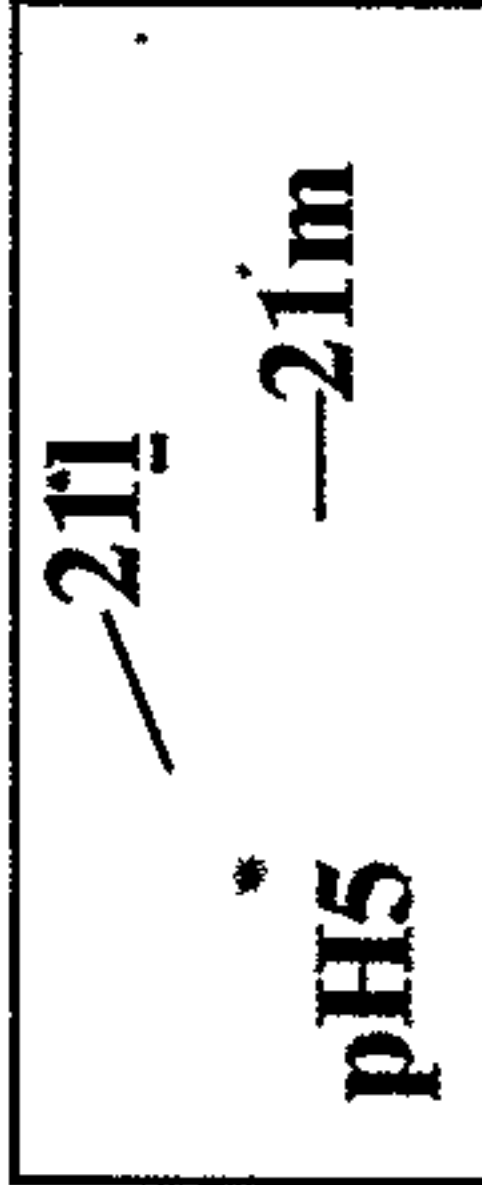


5.4C (cont)

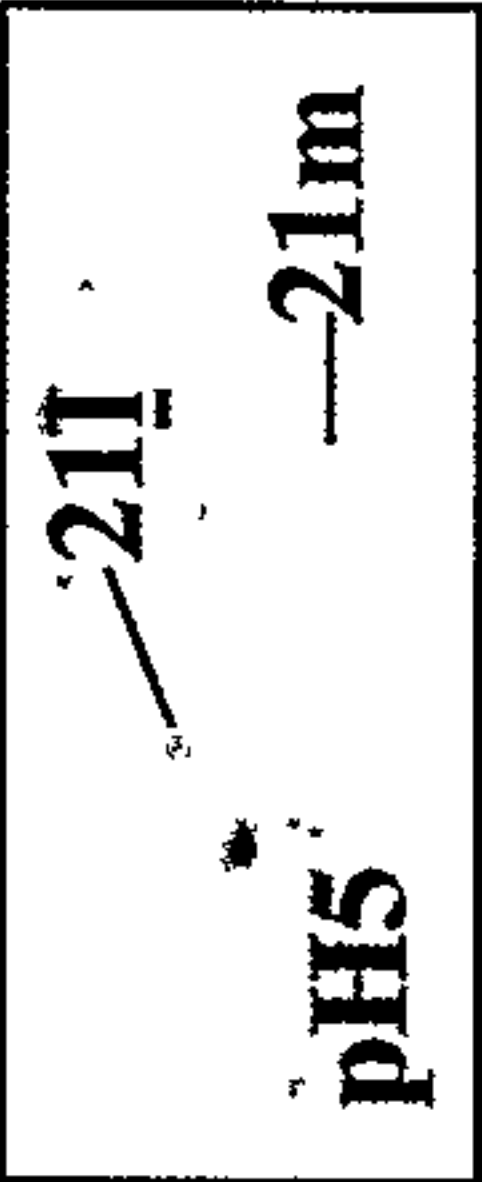
5.4.5 Chemostat 1



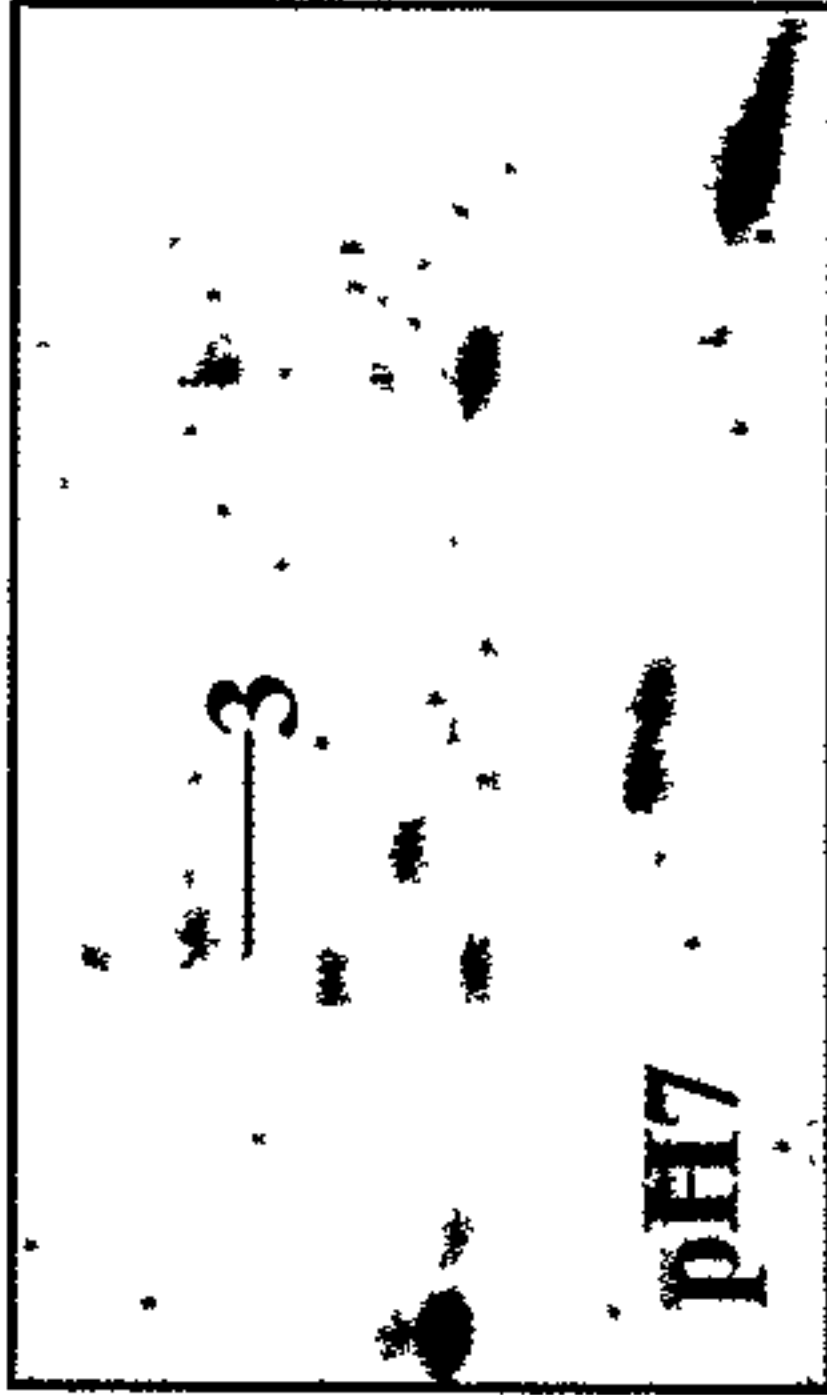
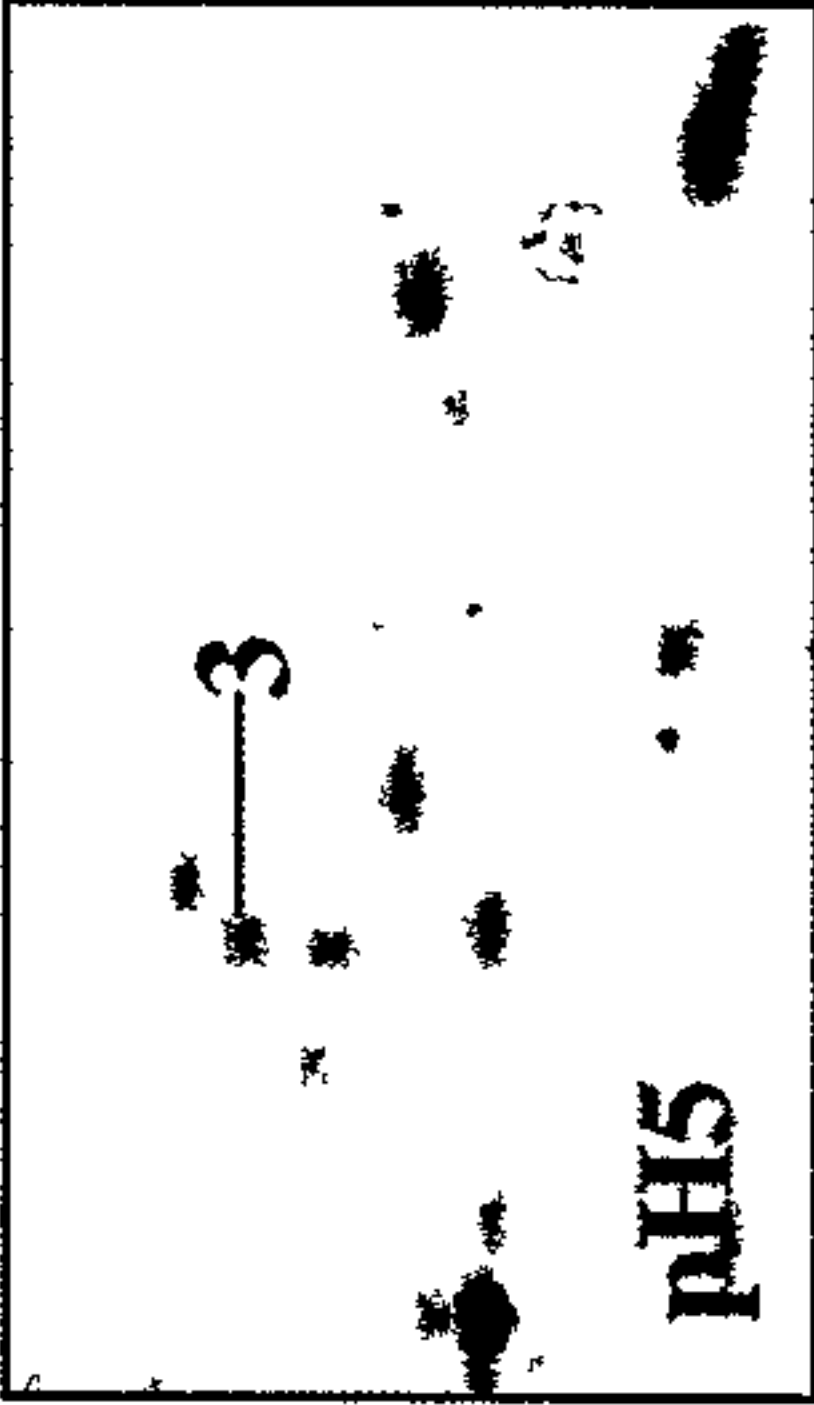
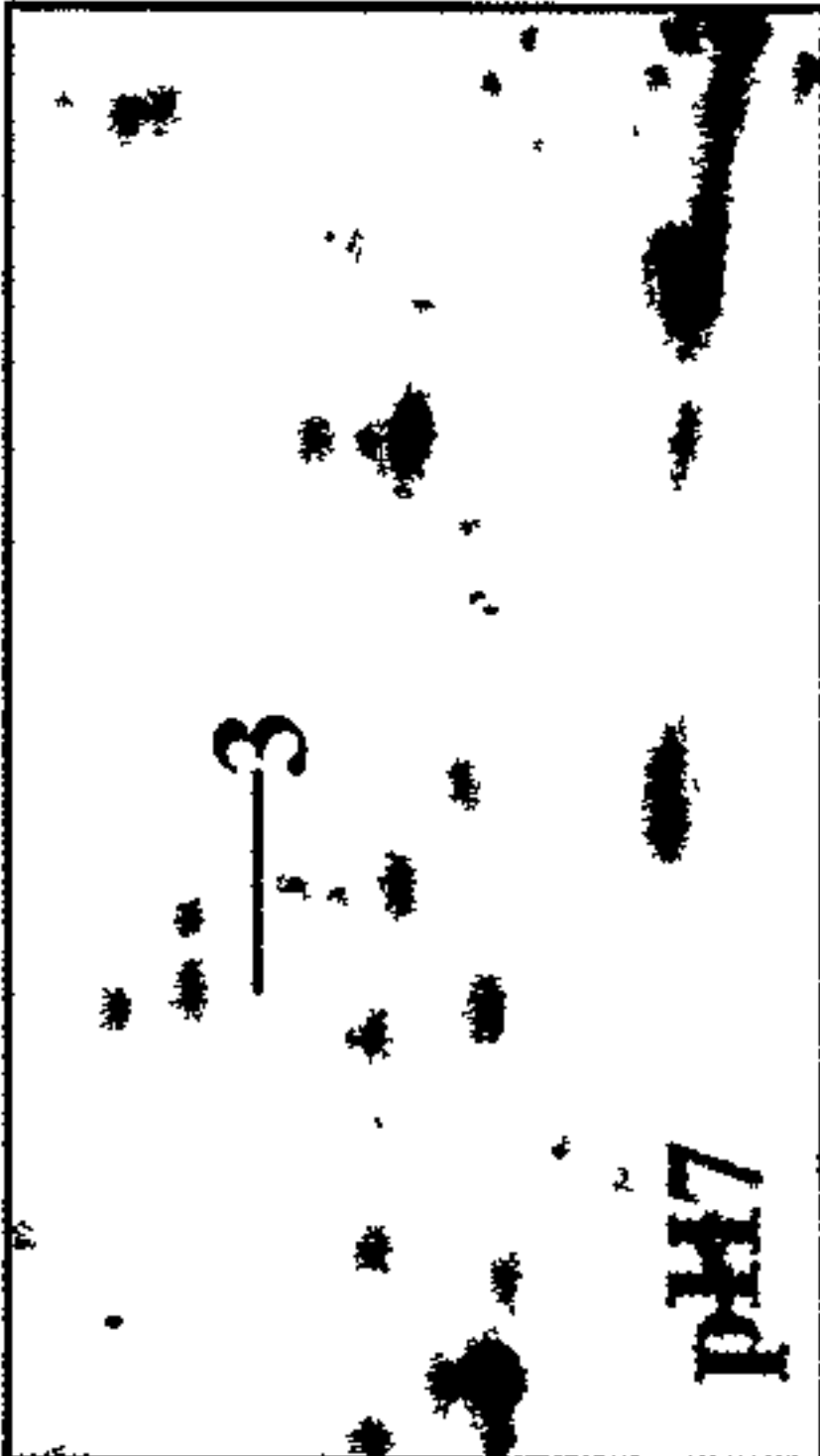
Chemostat 2



Chemostat 3



5.4.6



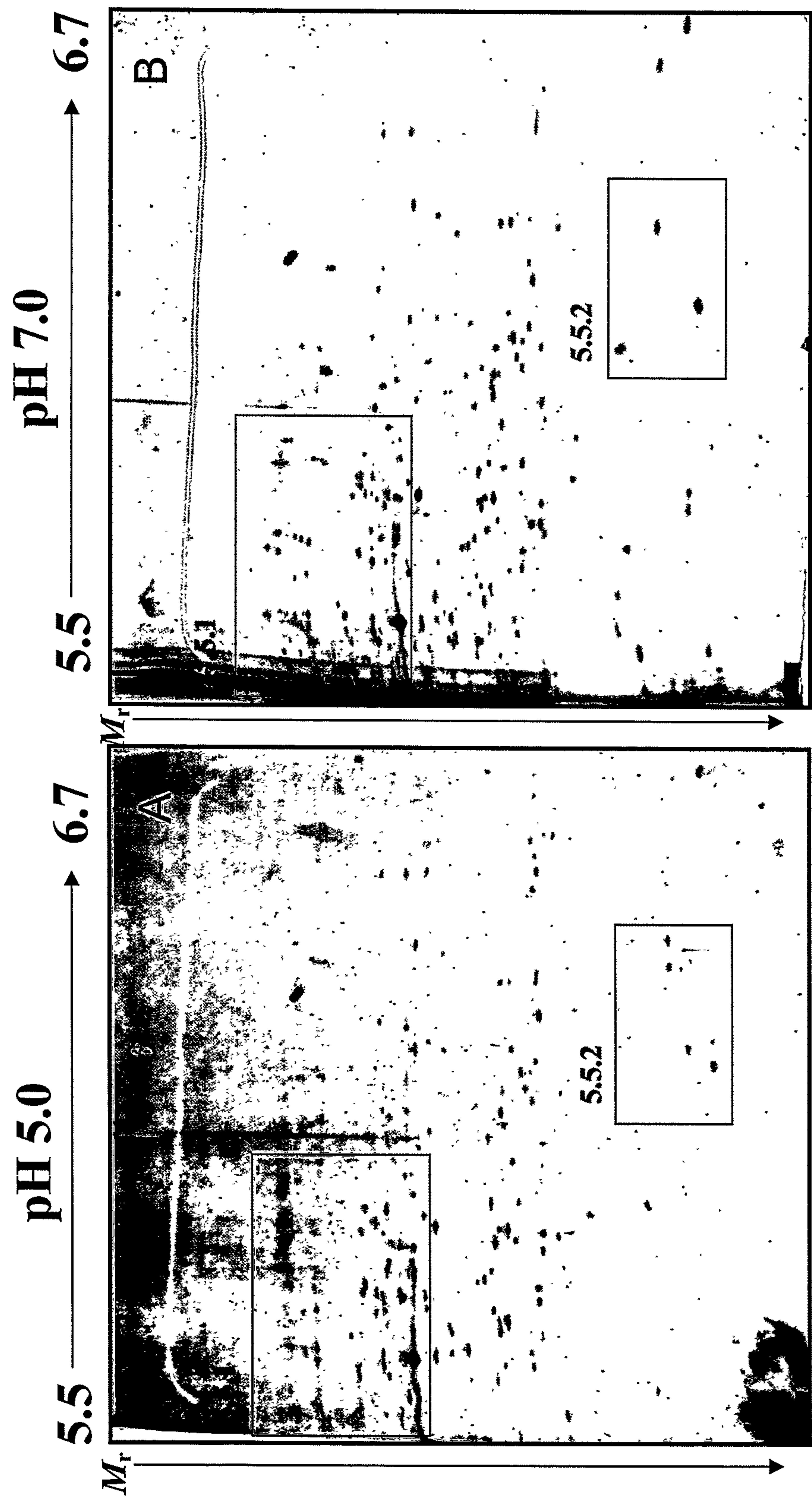
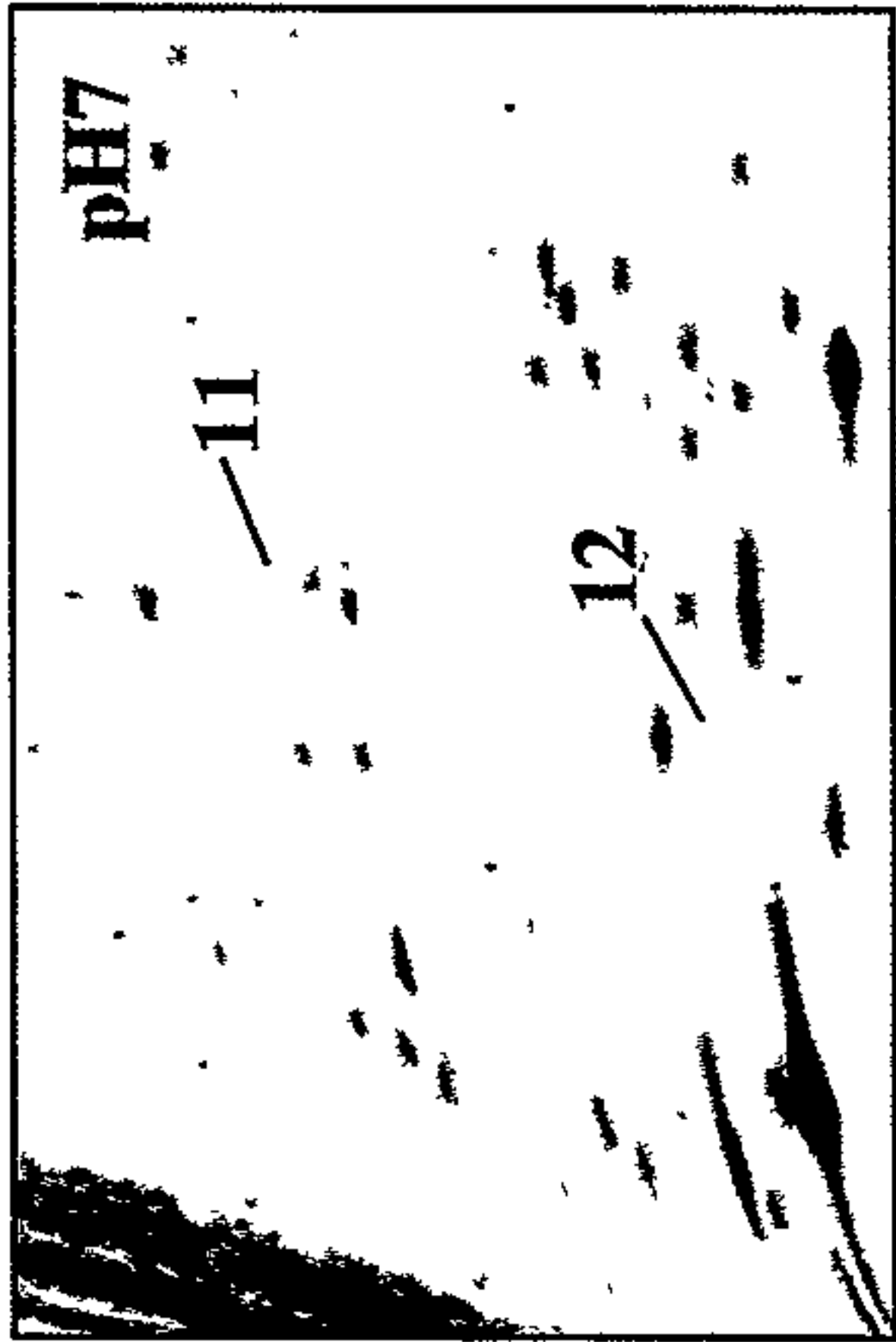
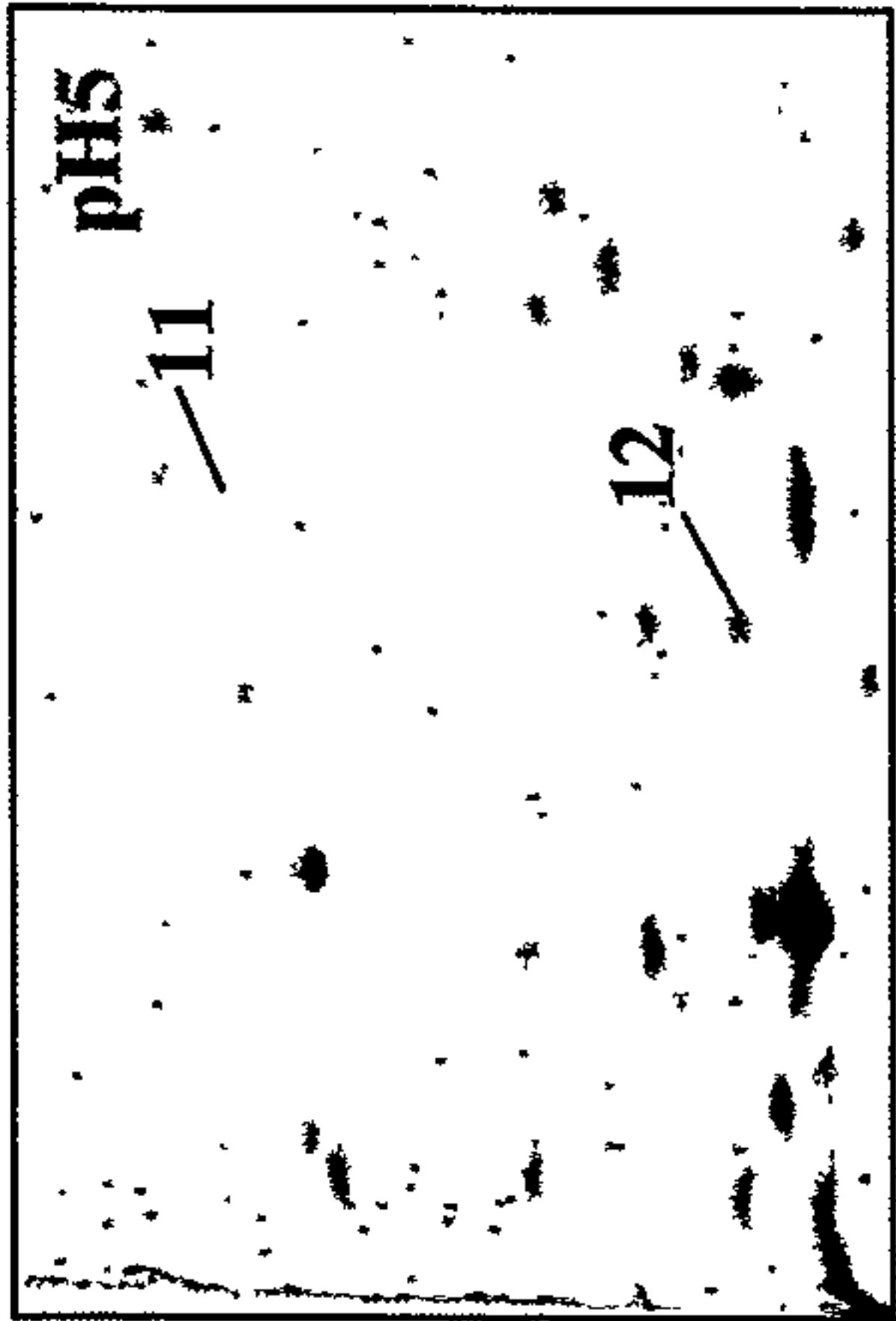


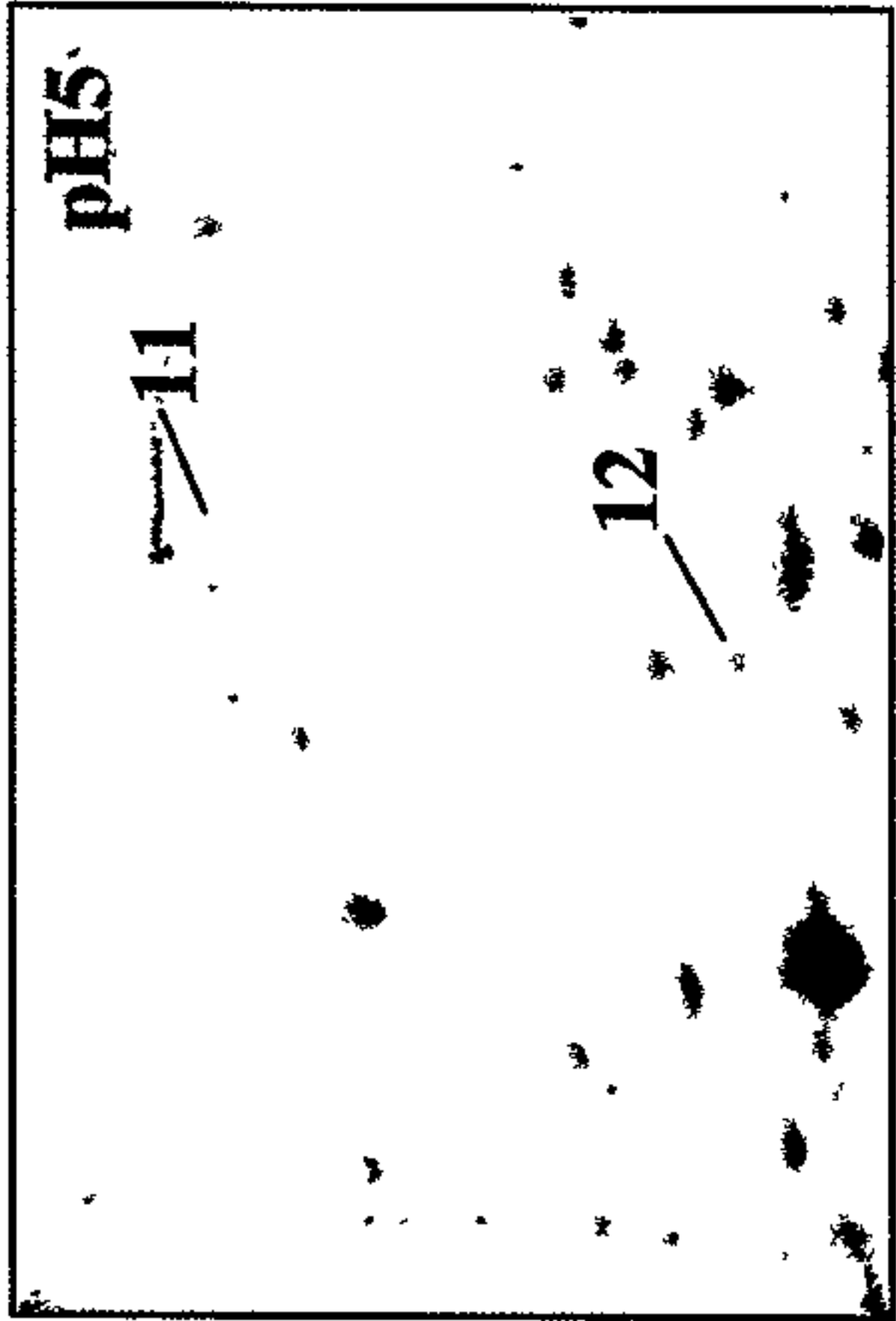
Figure 5.5. 2-DGE Sub-proteome maps (pH 5.5 – 6.7) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas 5.5.1 and 5.5.2, depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed area, corresponding to their respective chemostat 2-DGE's, are illustrated in 5.5C with spots numbers corresponding to those in Table 5.1.

5.5C

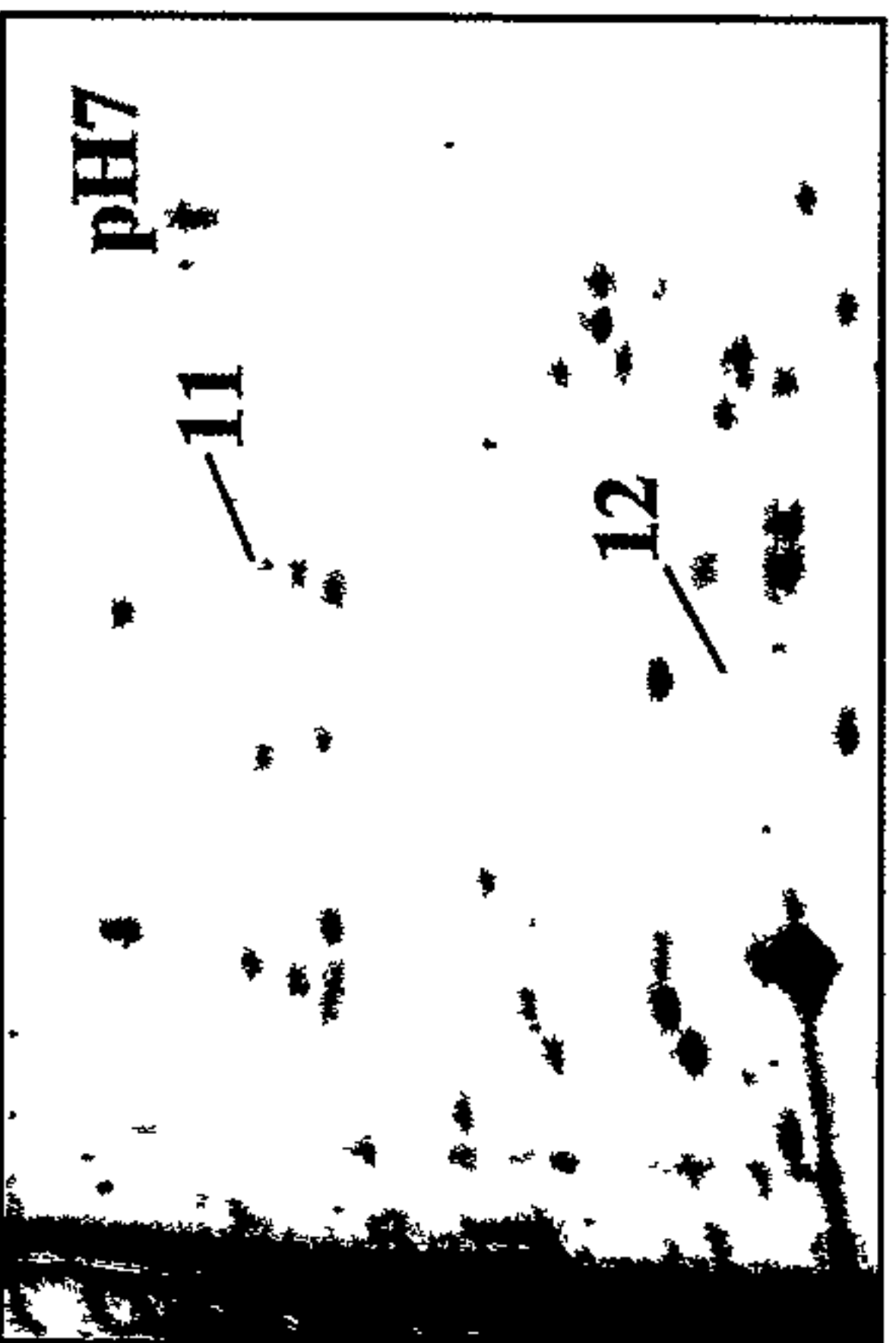
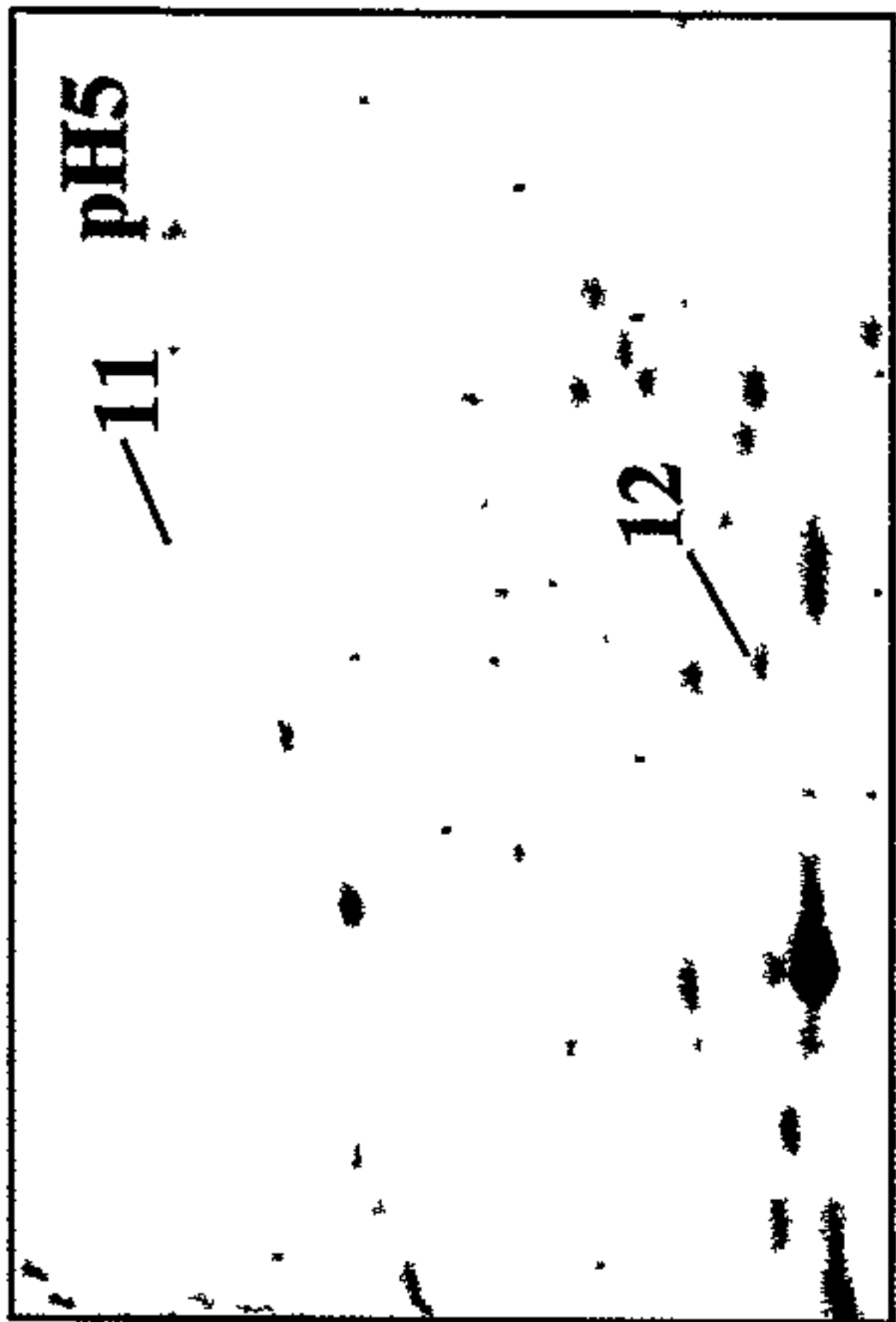
5.5.1 Chemostat 1



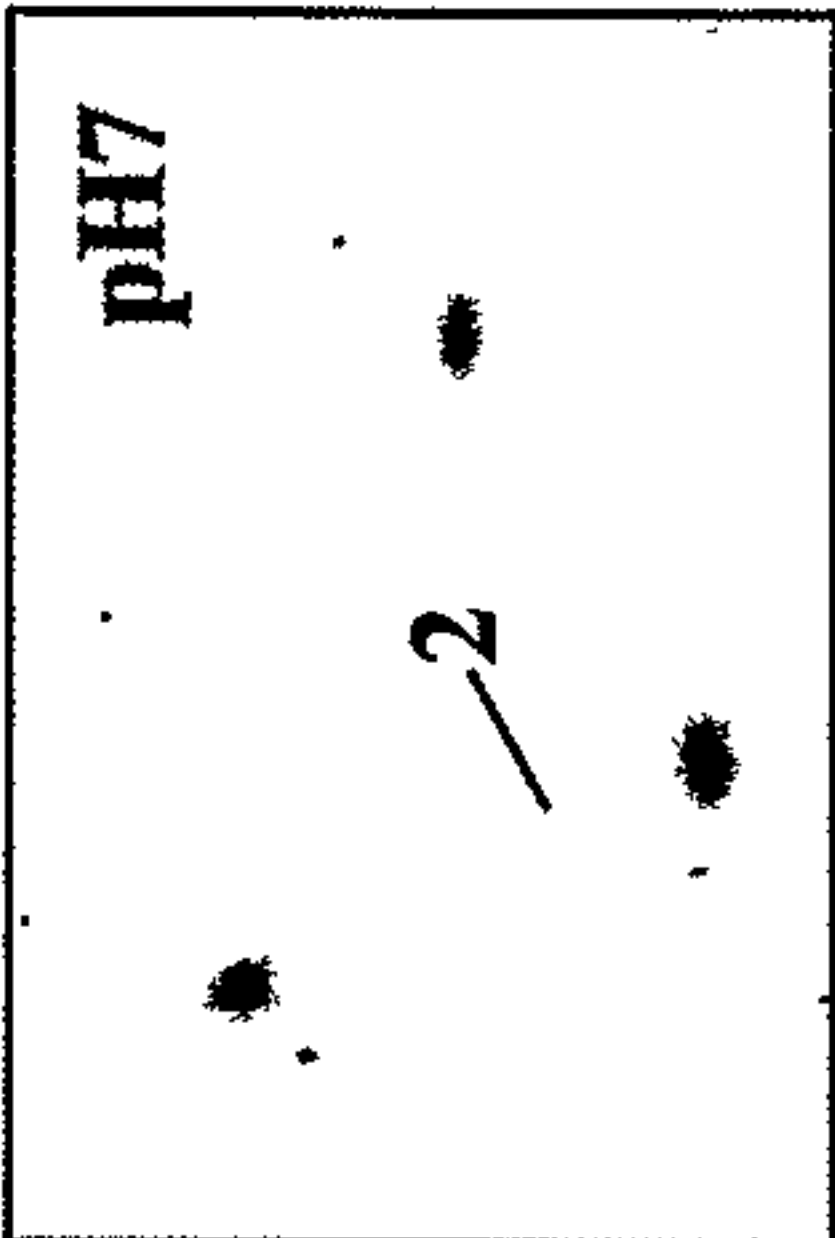
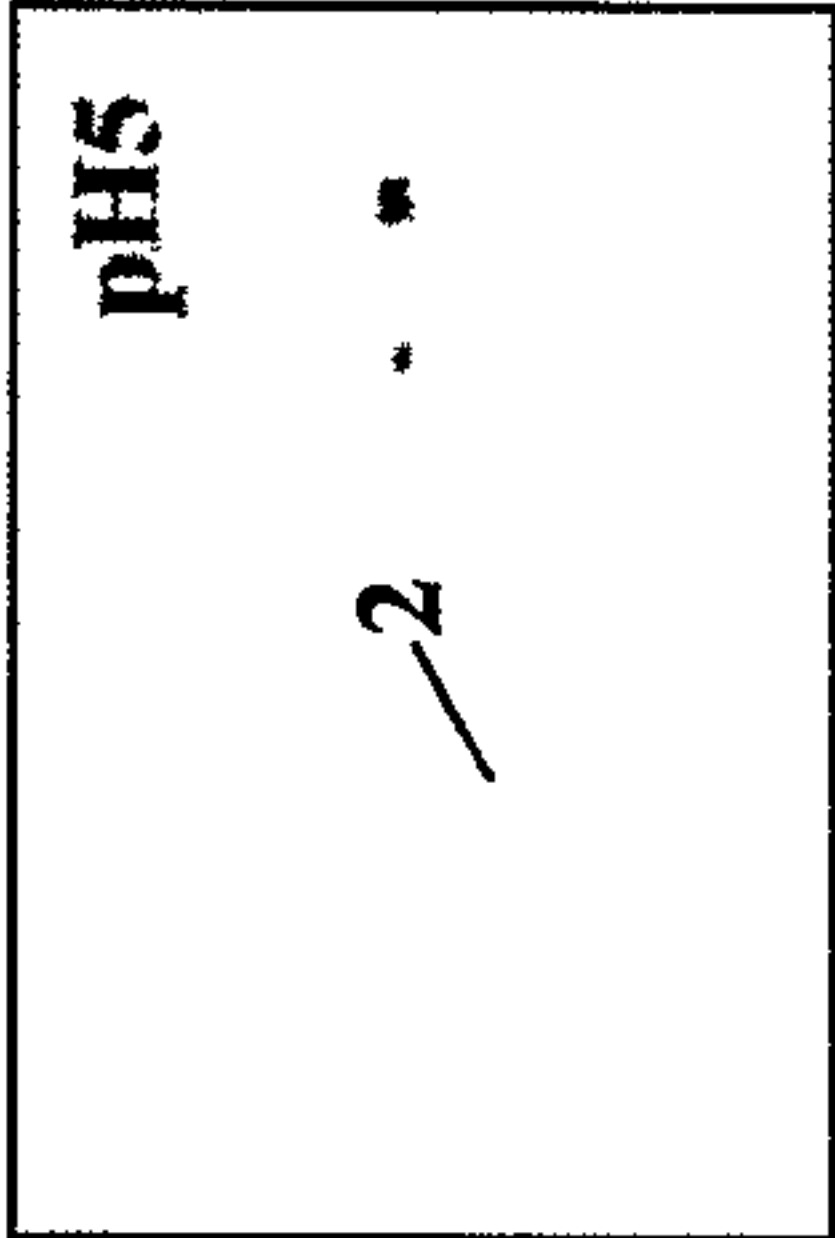
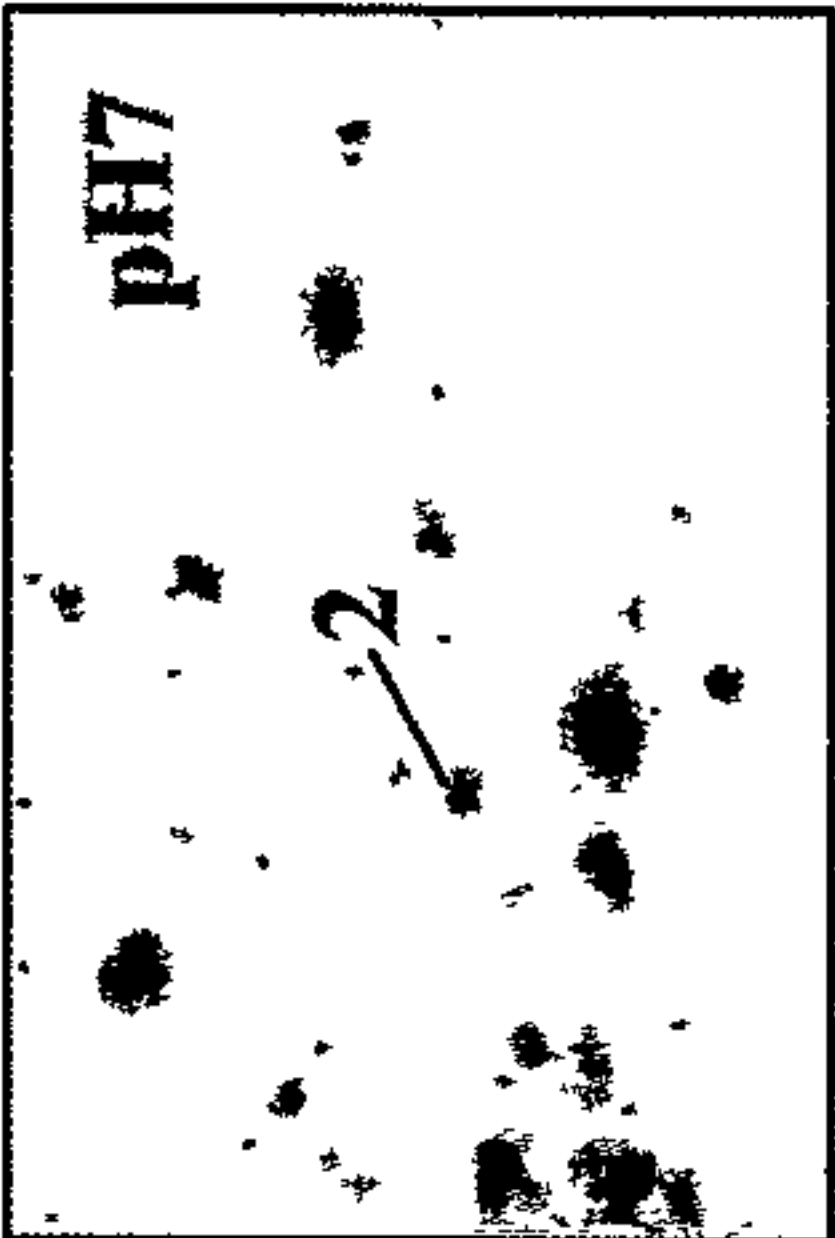
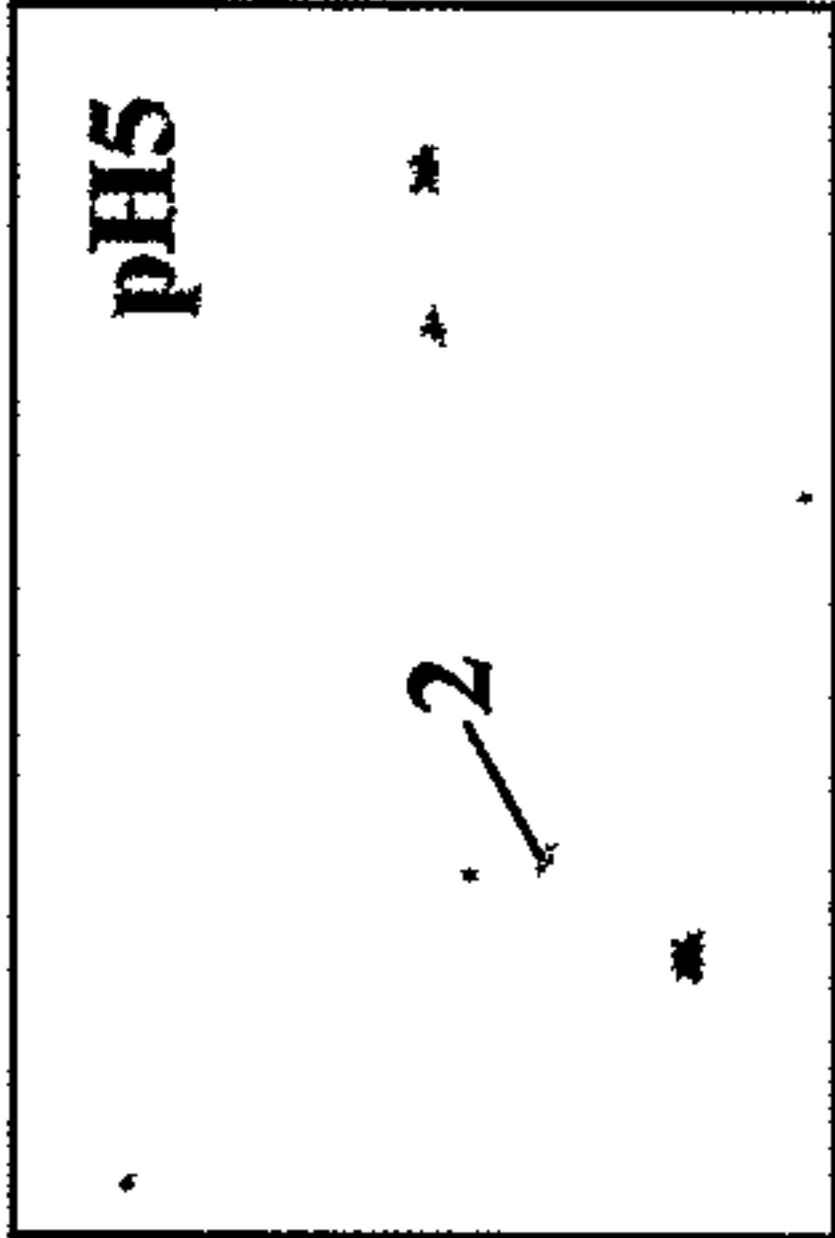
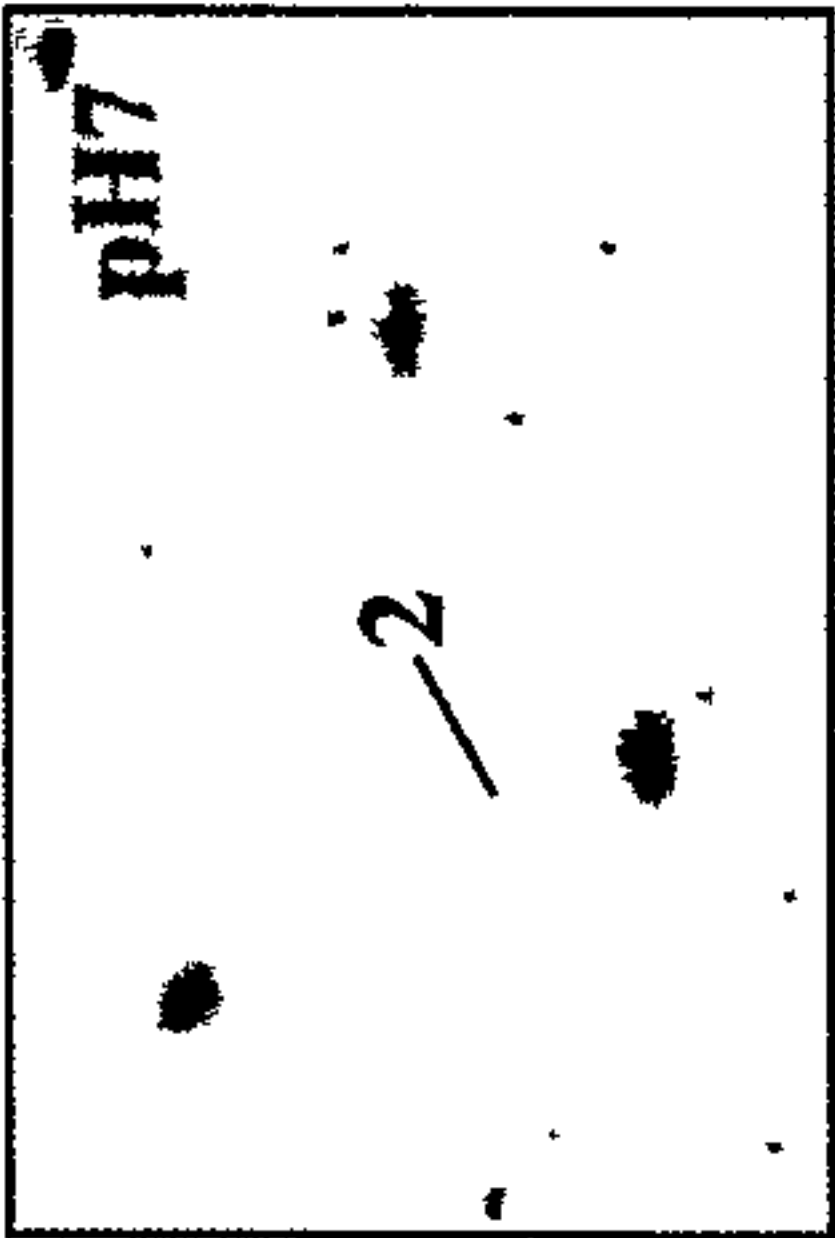
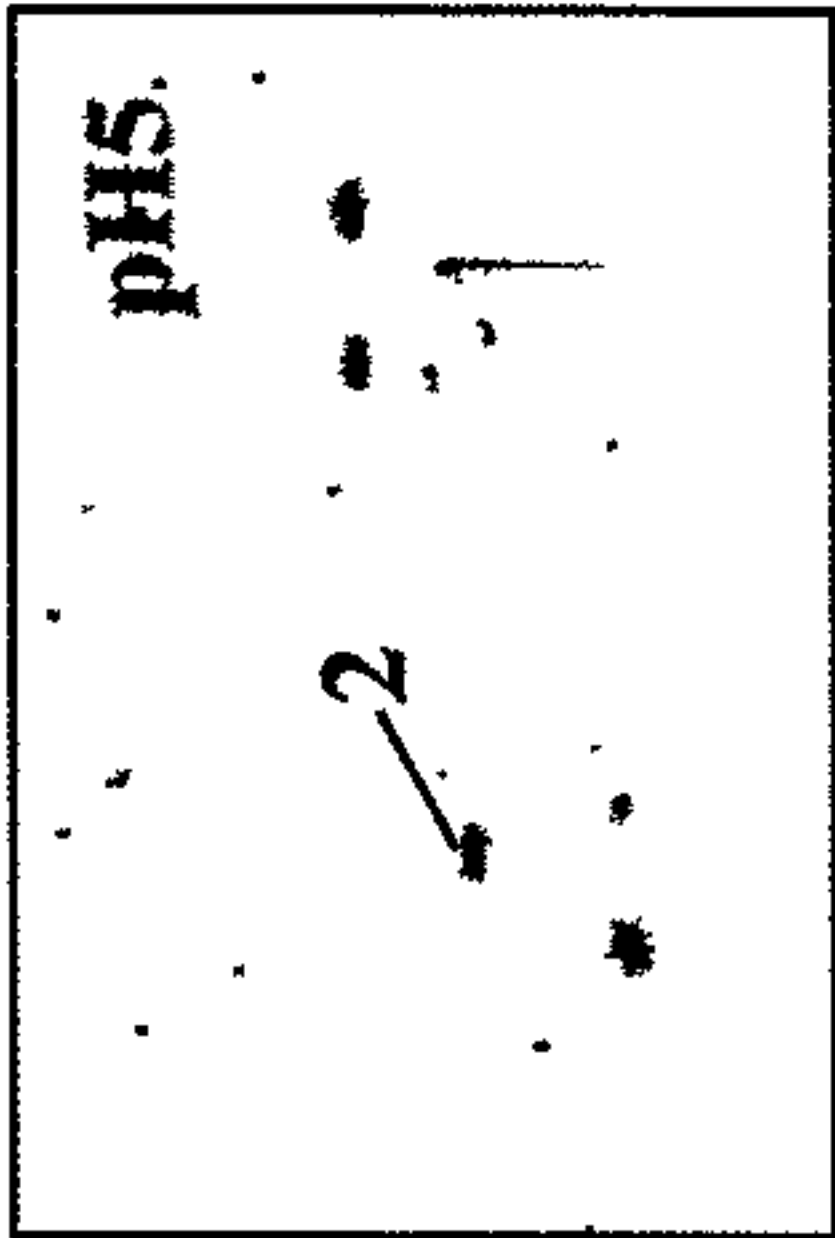
Chemostat 2



Chemostat 3



5.5.2



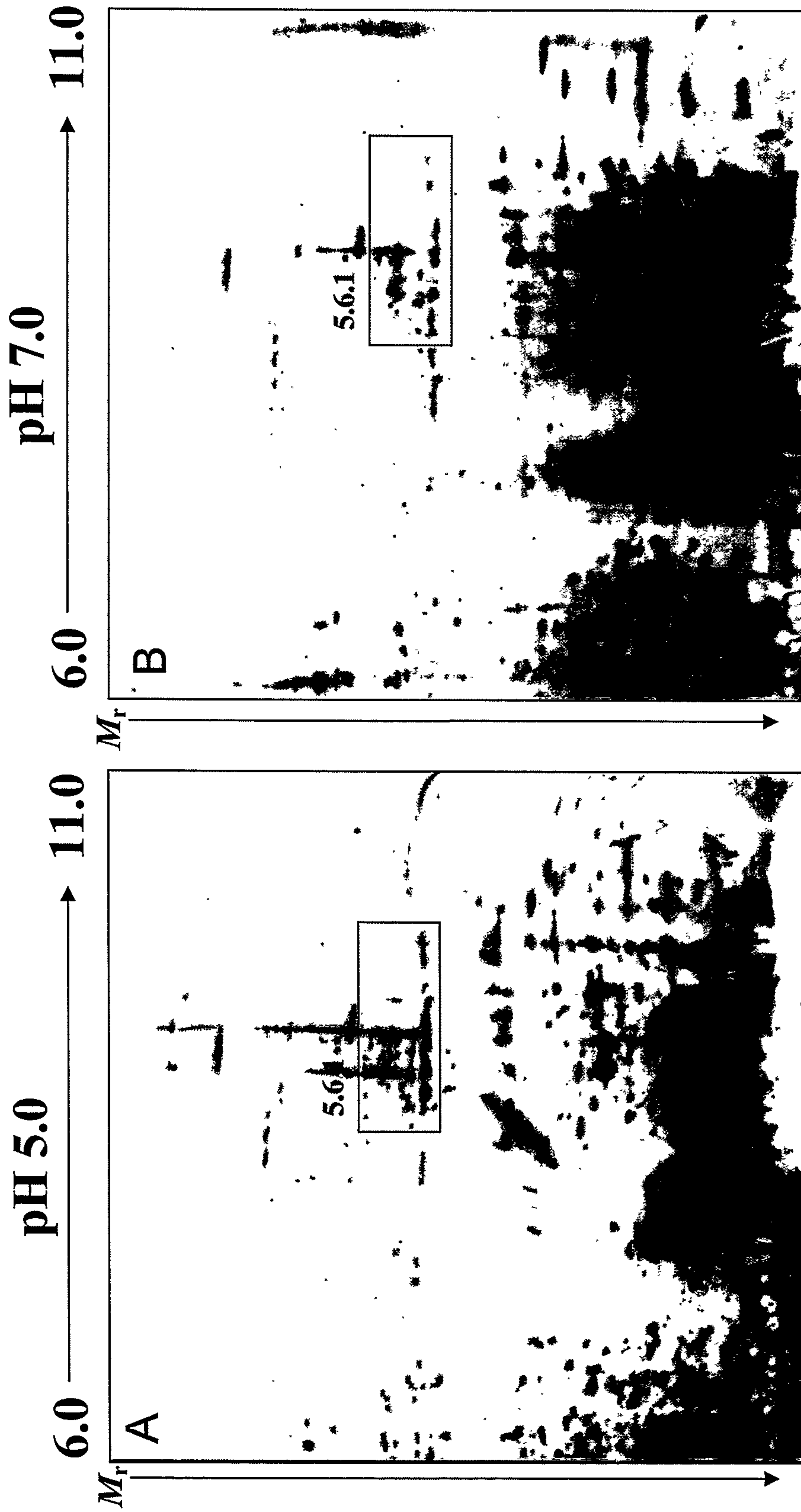
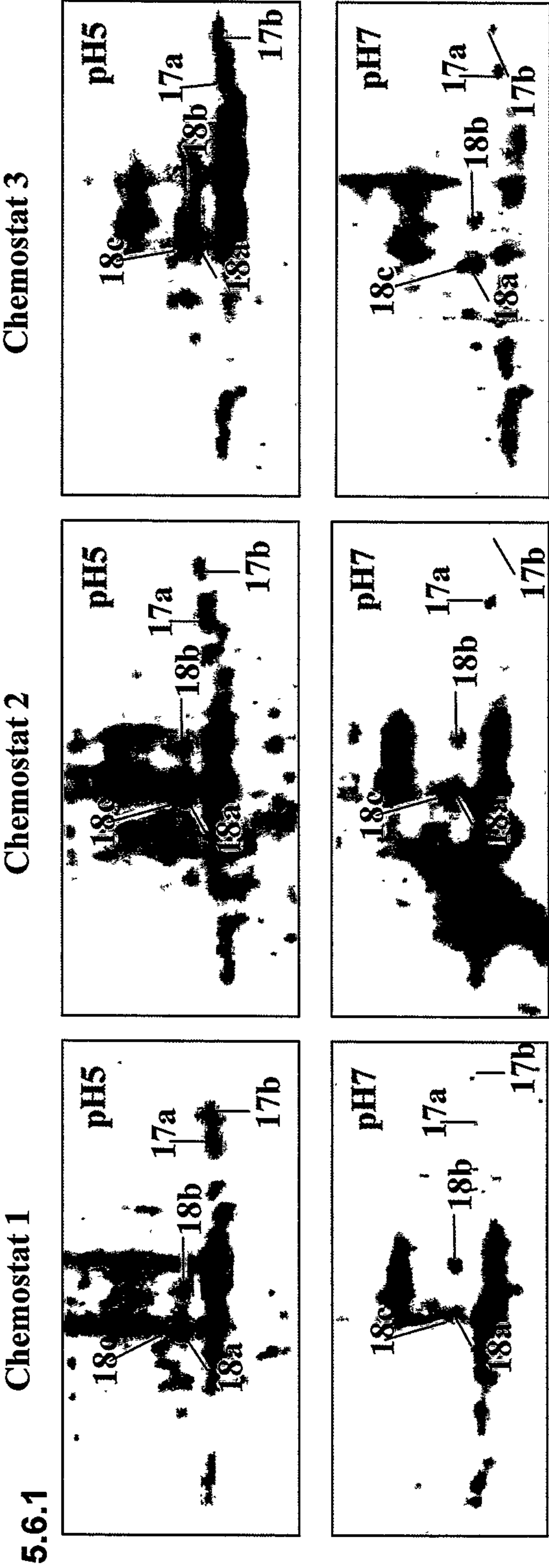


Figure 5.6. 2-DGE Sub-proteome maps (pH 6.0 – 11.0) of *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed area 5.6.1, depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed area, corresponding to their respective chemostat 2-DGEs, are illustrated in 5.6C with spot numbers corresponding to those in Table 5.1.

5.6C



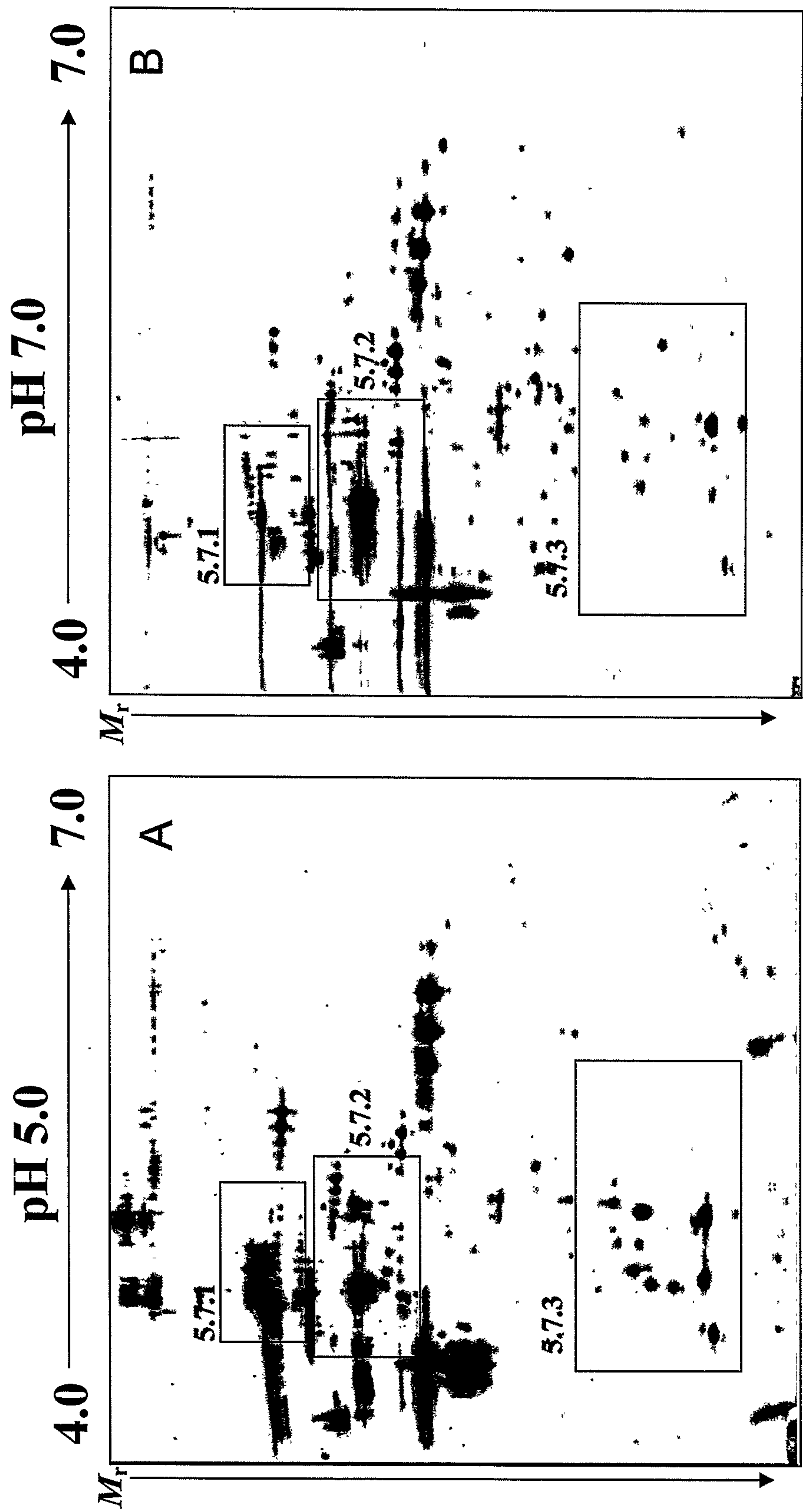
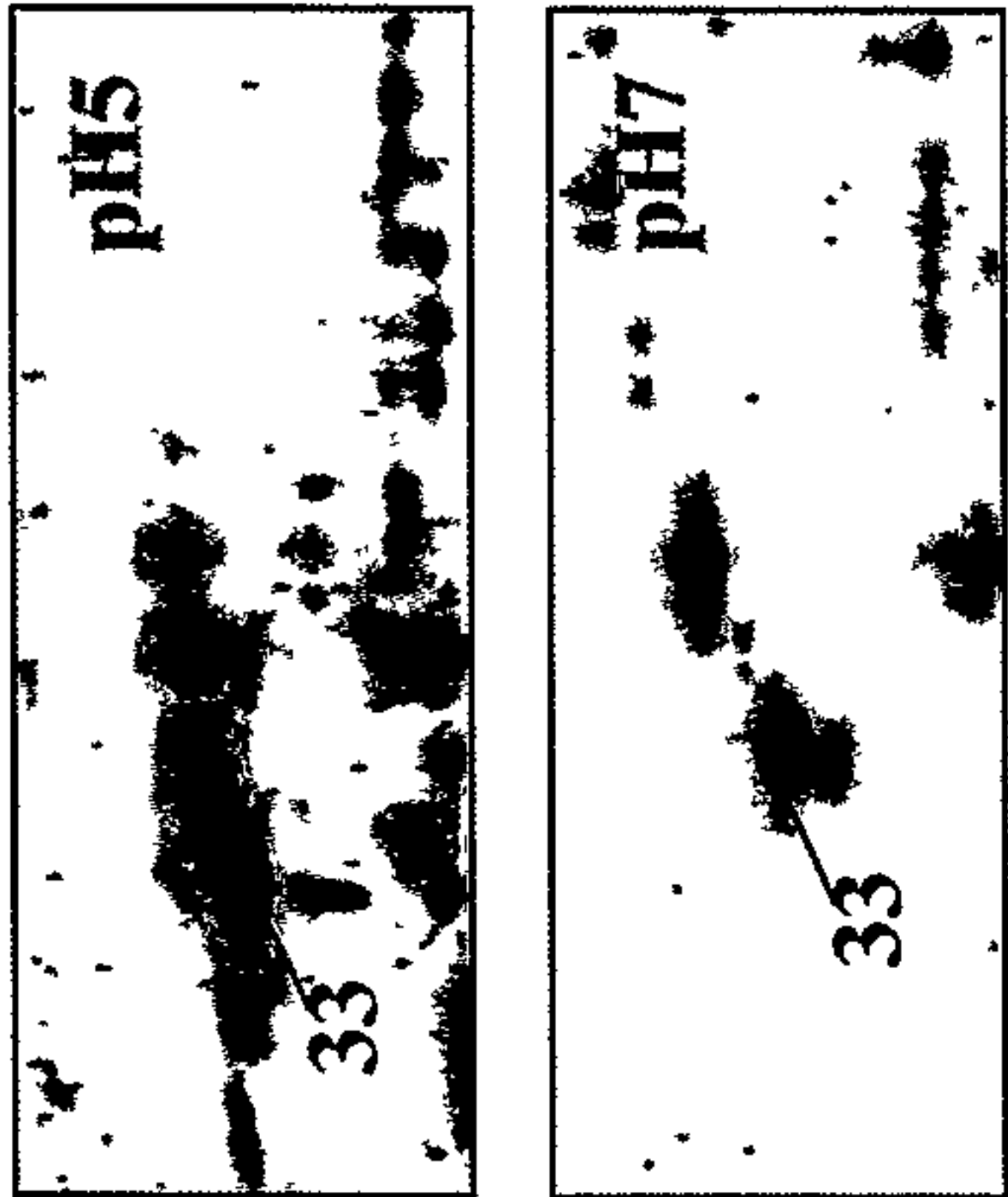


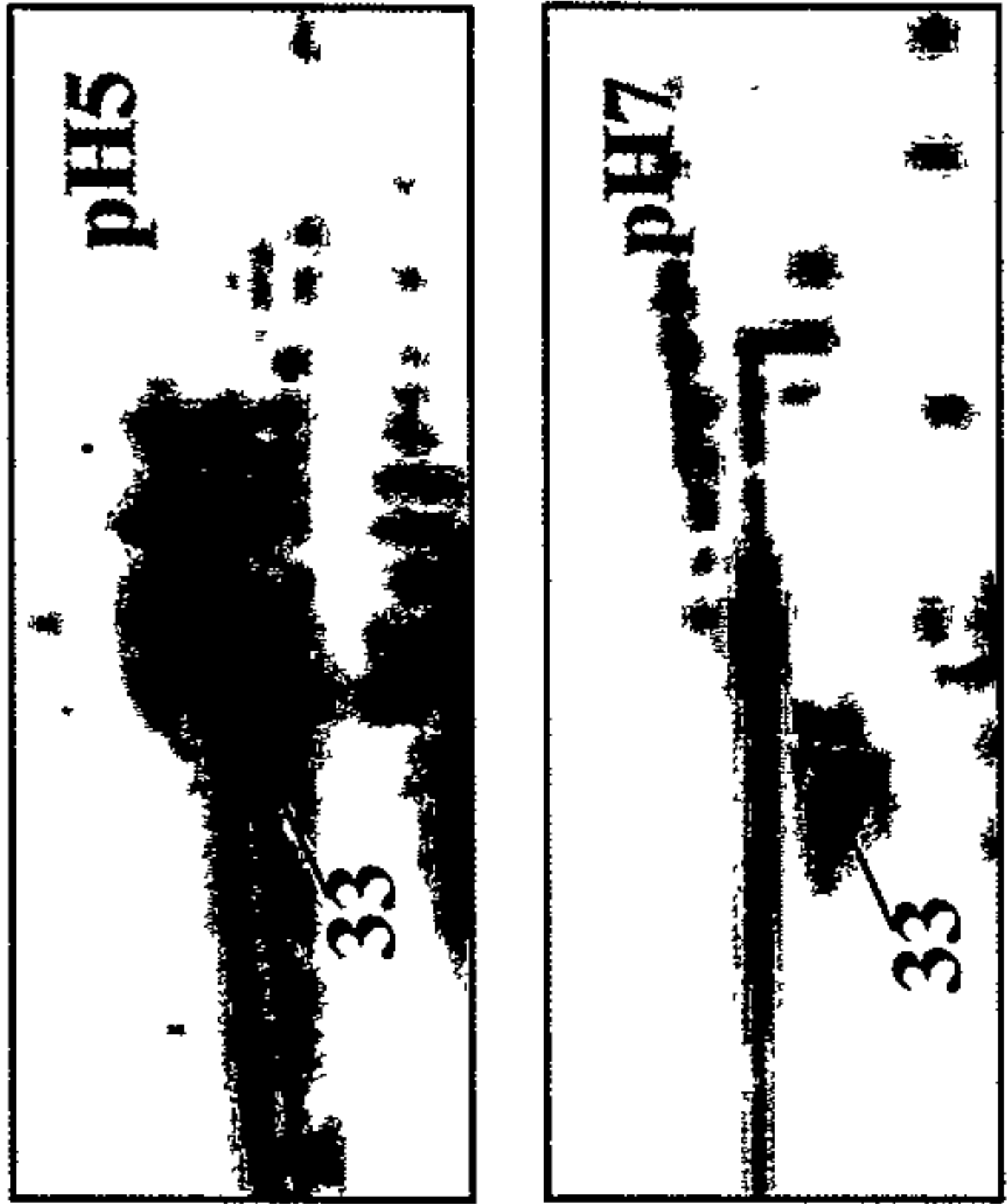
Figure 5.7. 2-DGE Sub-proteome maps (pH 6.0 – 11.0) of the extracellular culture fluid from *S. mutans* LT11 grown at pH 5.0 (A) and pH 7.0 (B). Boxed areas (5.7.1, 5.7.2, 5.7.3) depict spot/s that is/are differentially expressed. Enlarged triplicates of boxed areas, corresponding to their respective chemostat 2-DGEs, are illustrated in 5.7C spot numbers corresponding to those in table 5.2.

5.7C

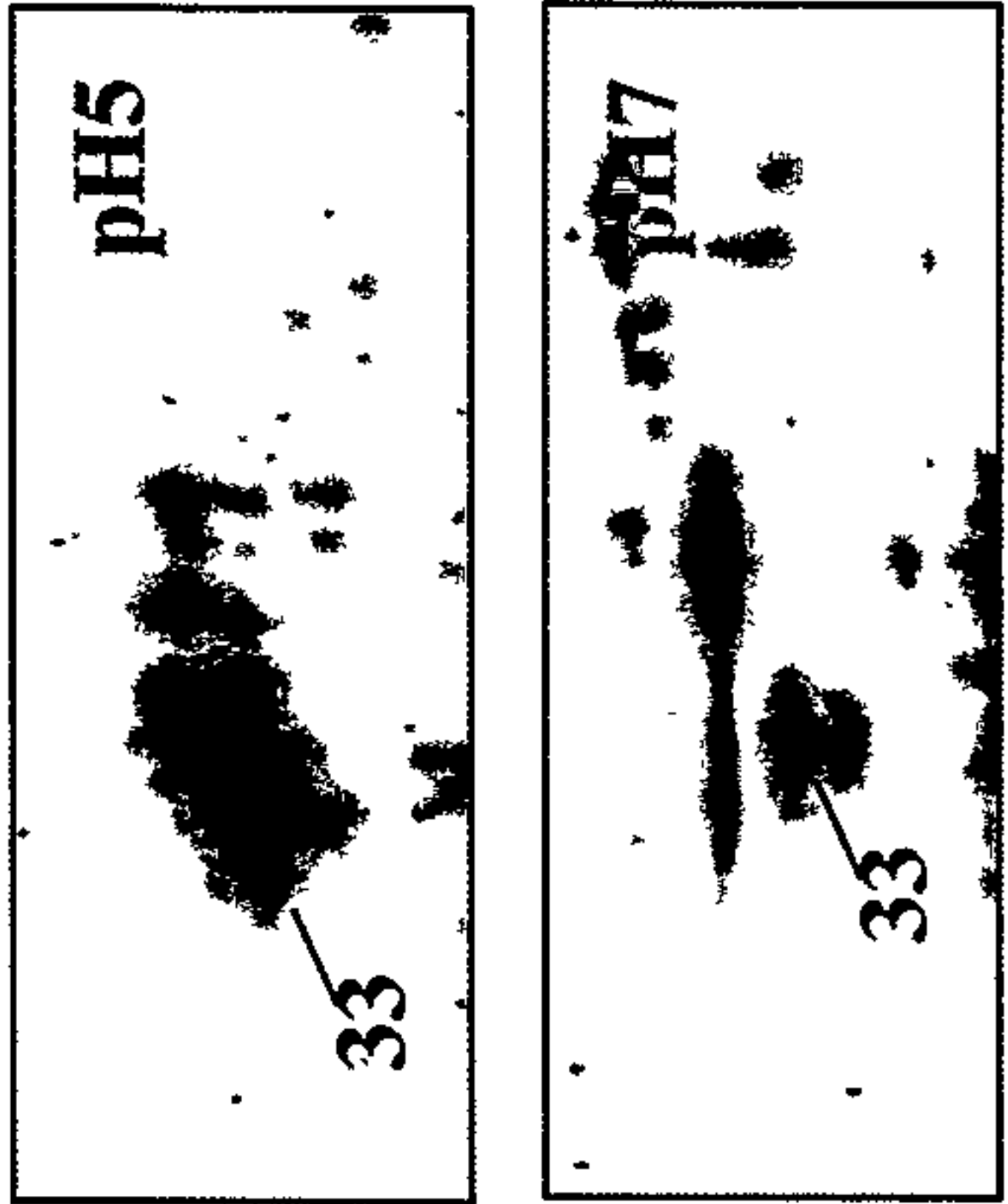
5.7.1 Chemostat 1



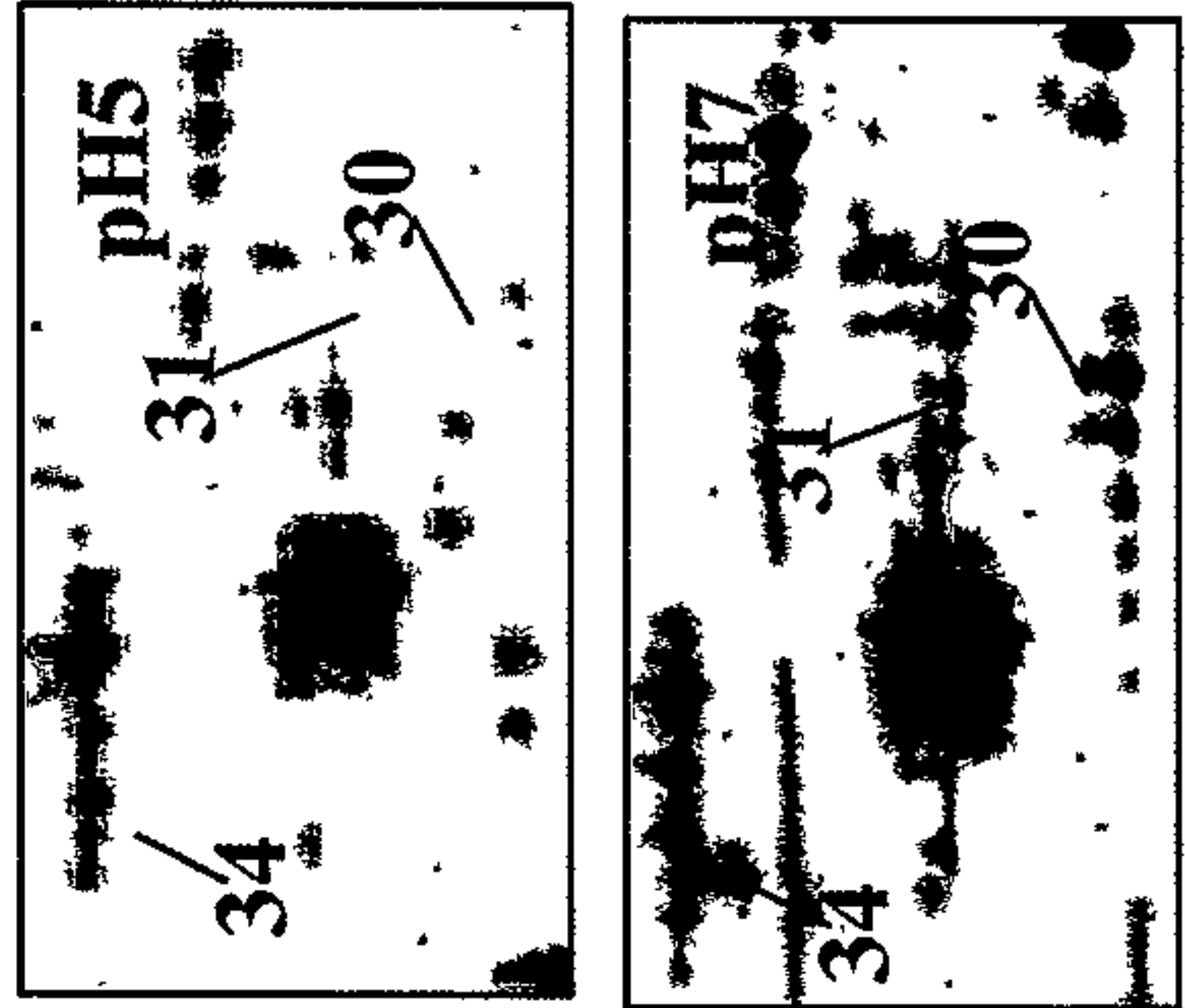
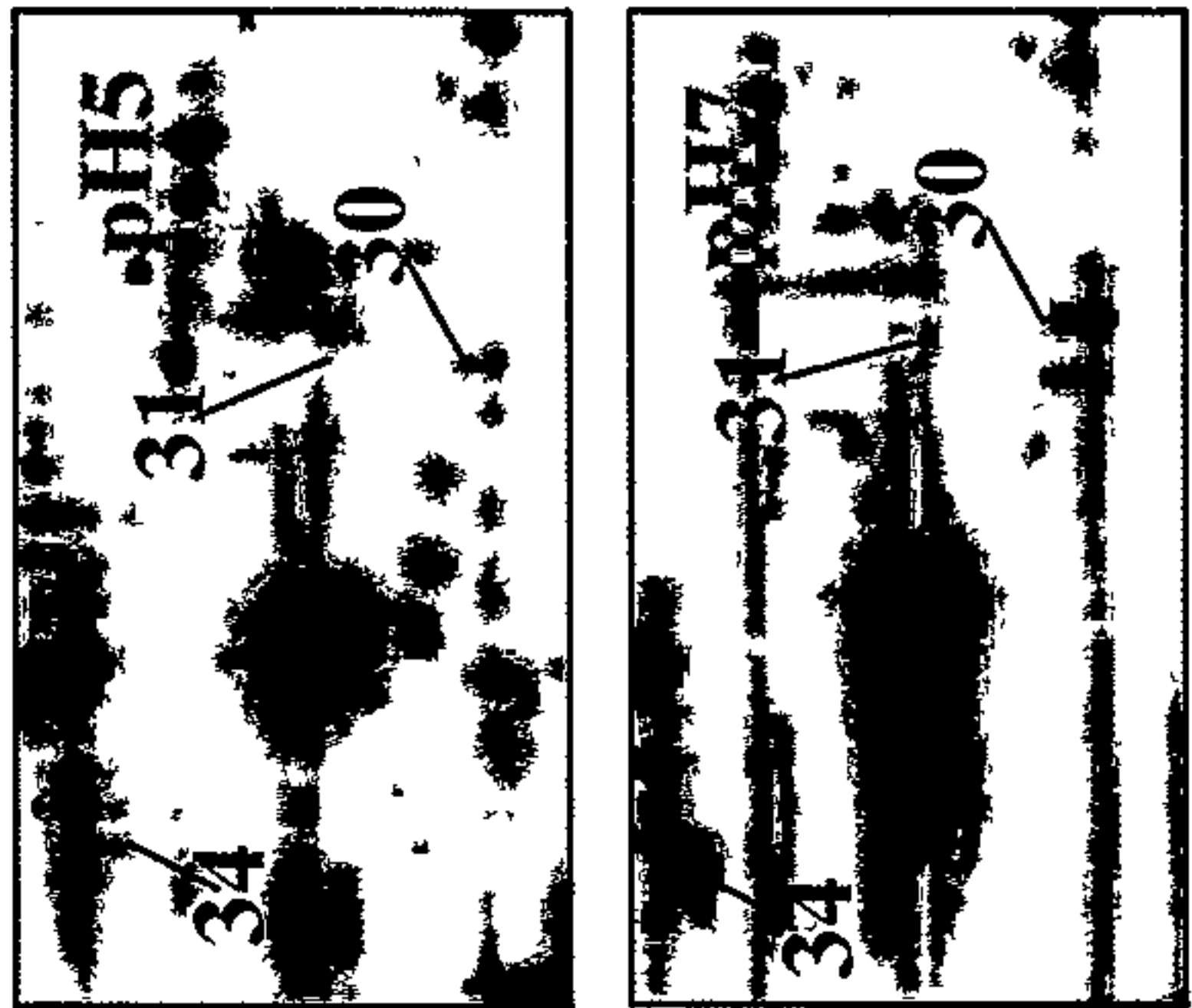
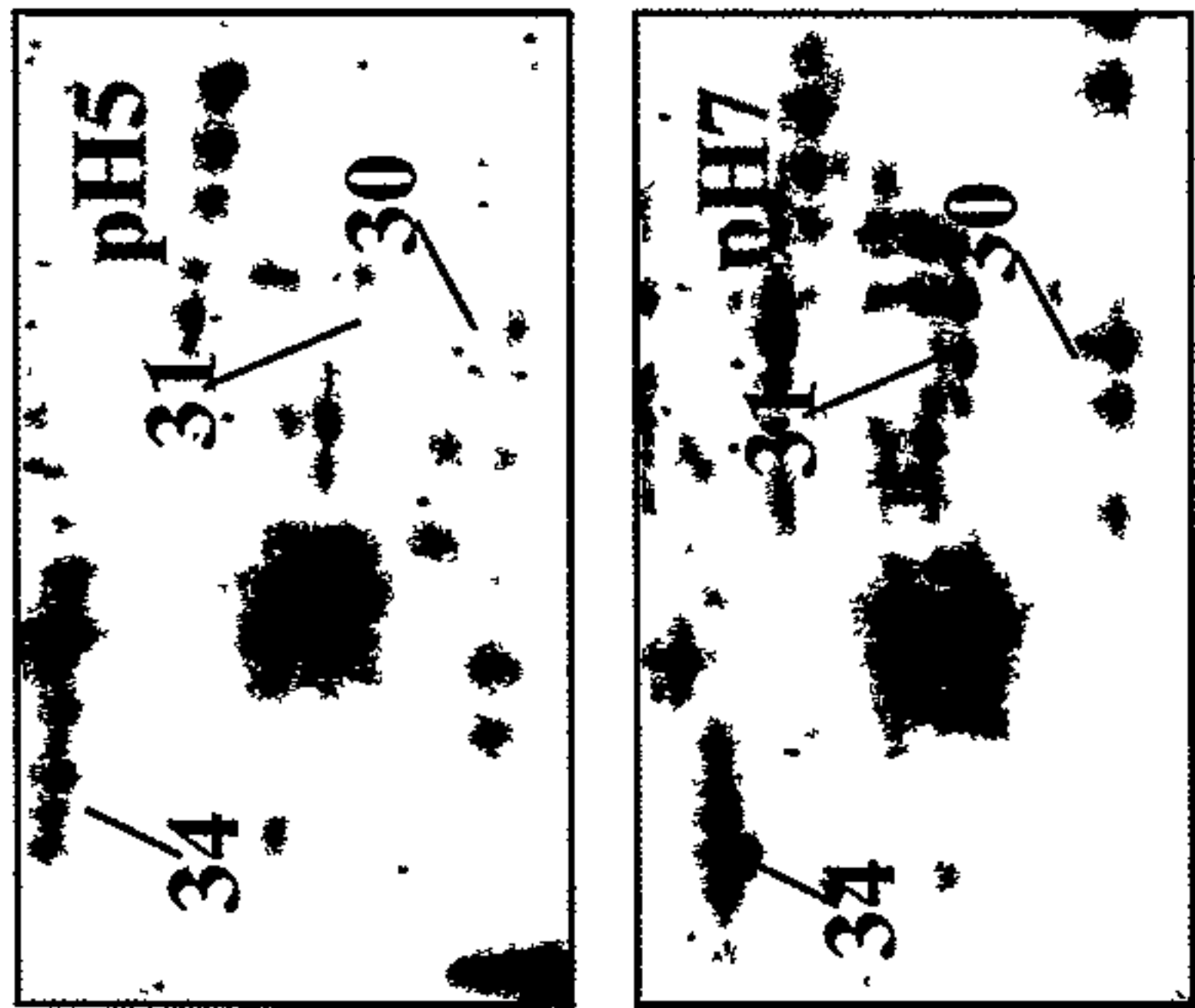
Chemostat 2



Chemostat 3



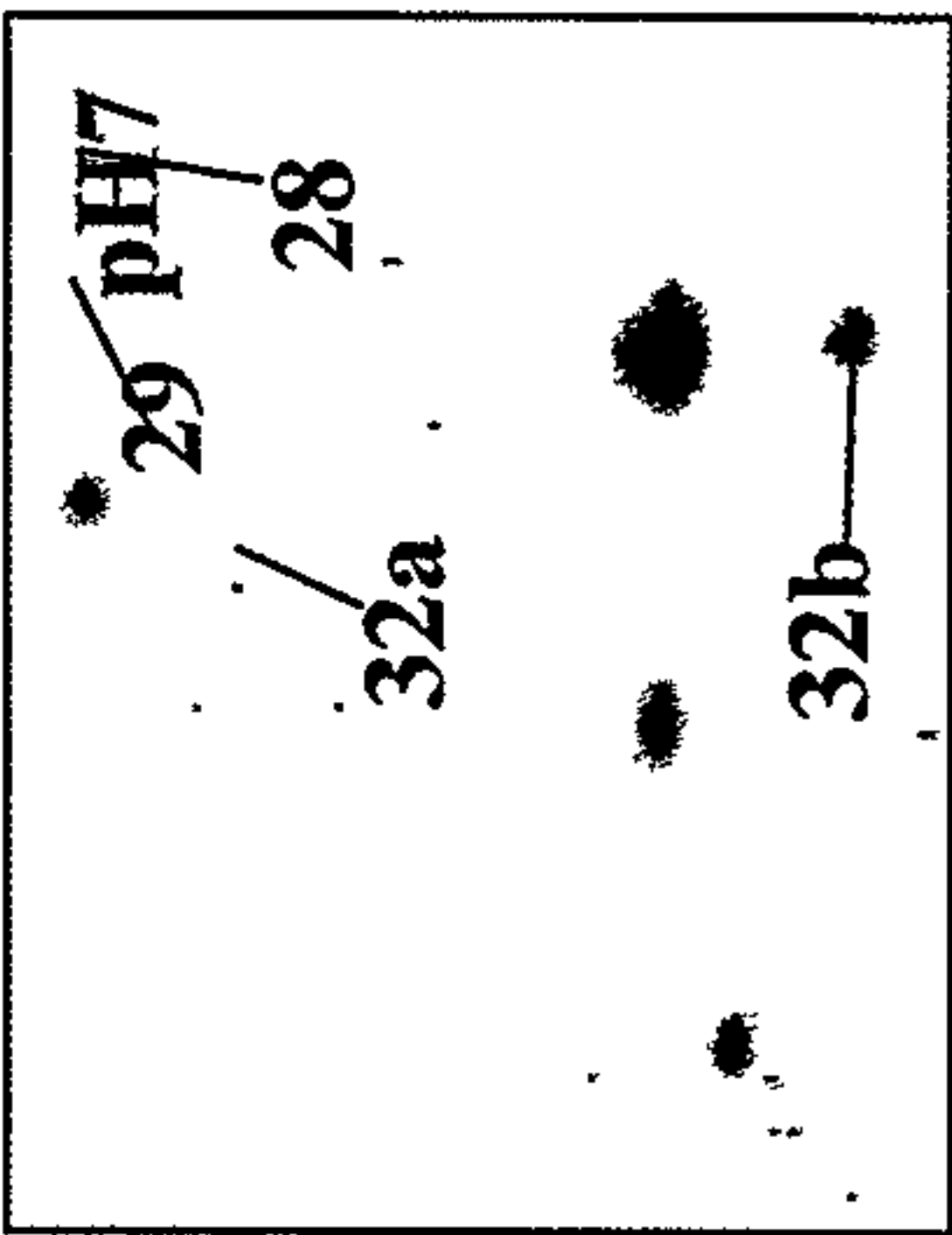
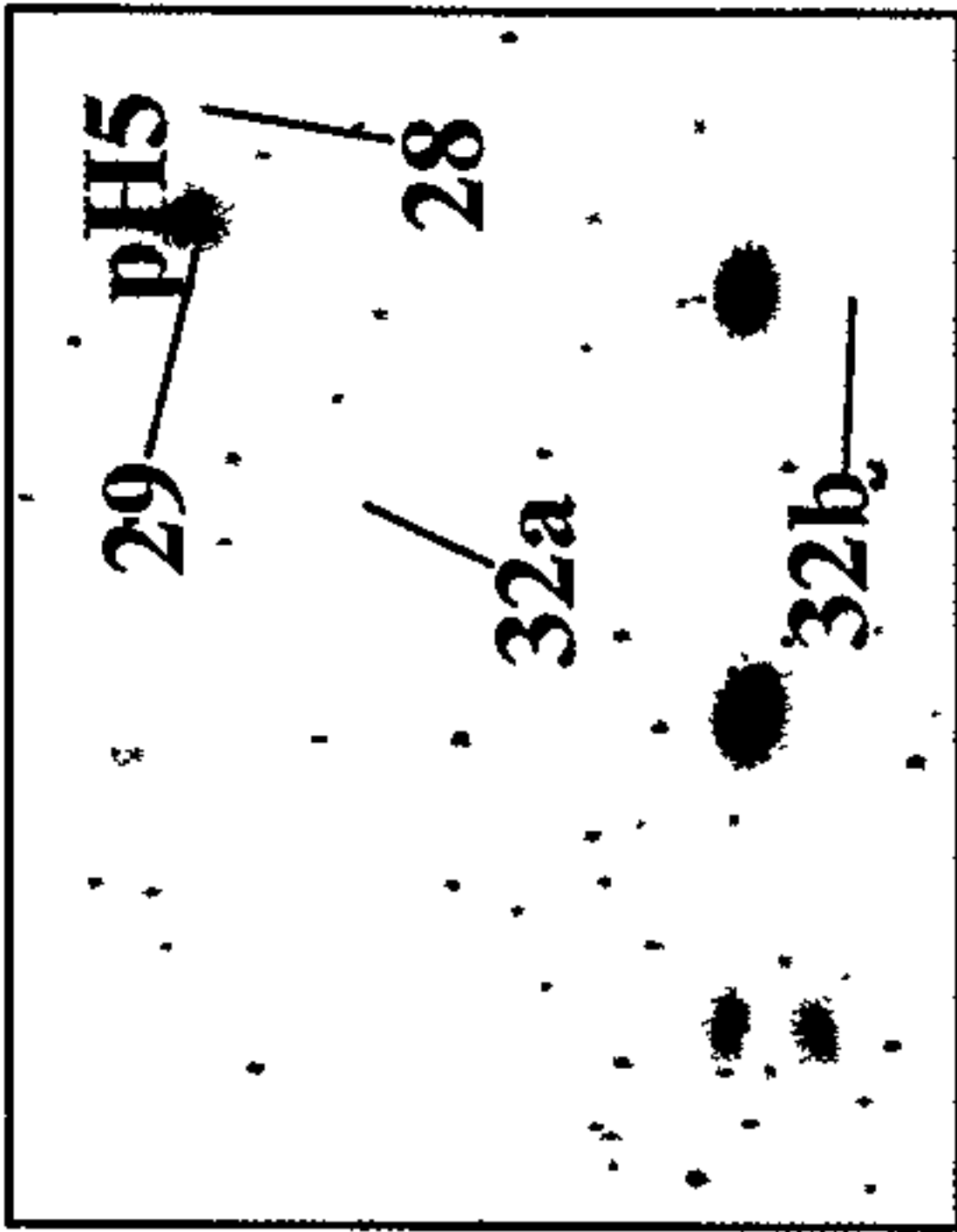
5.7.2



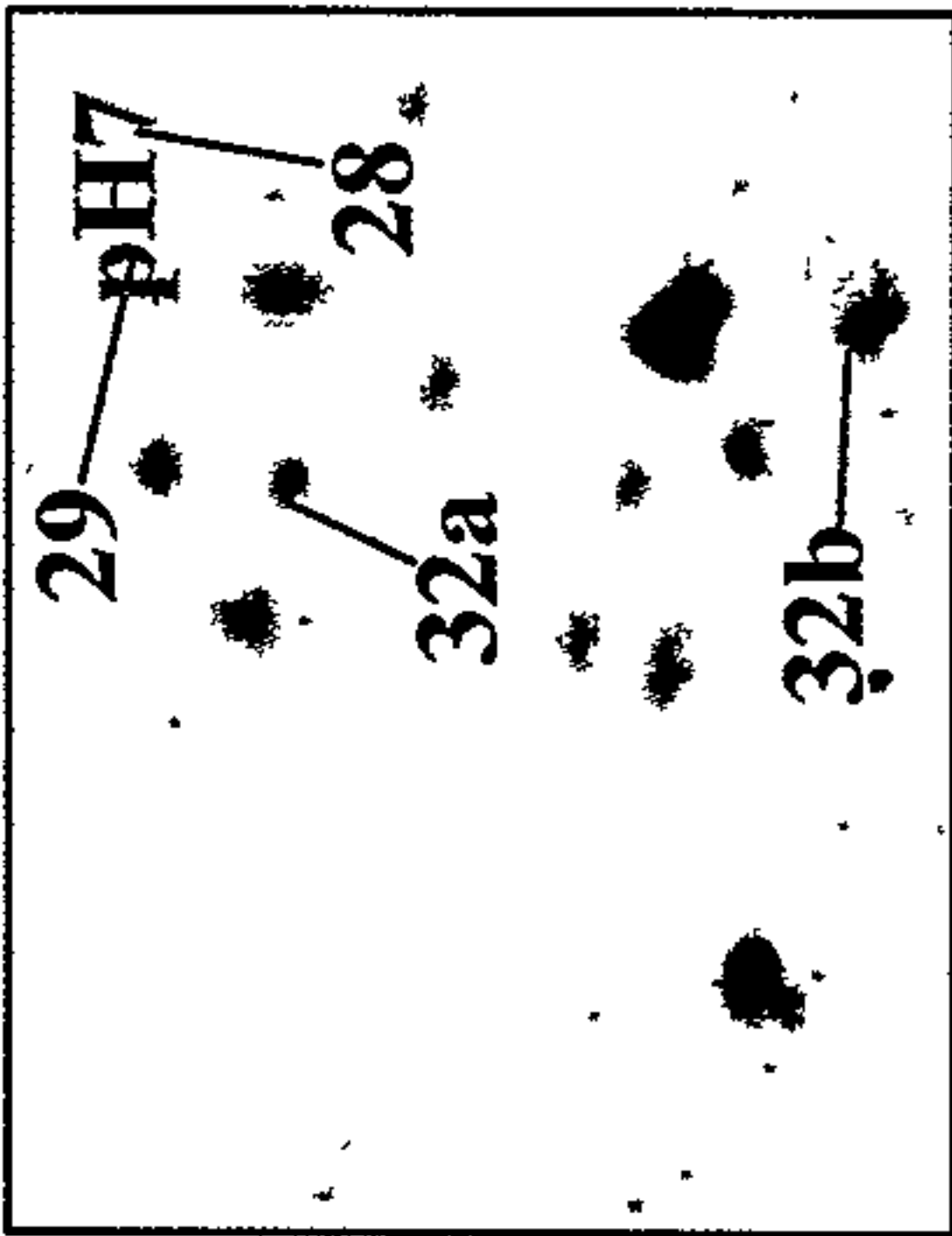
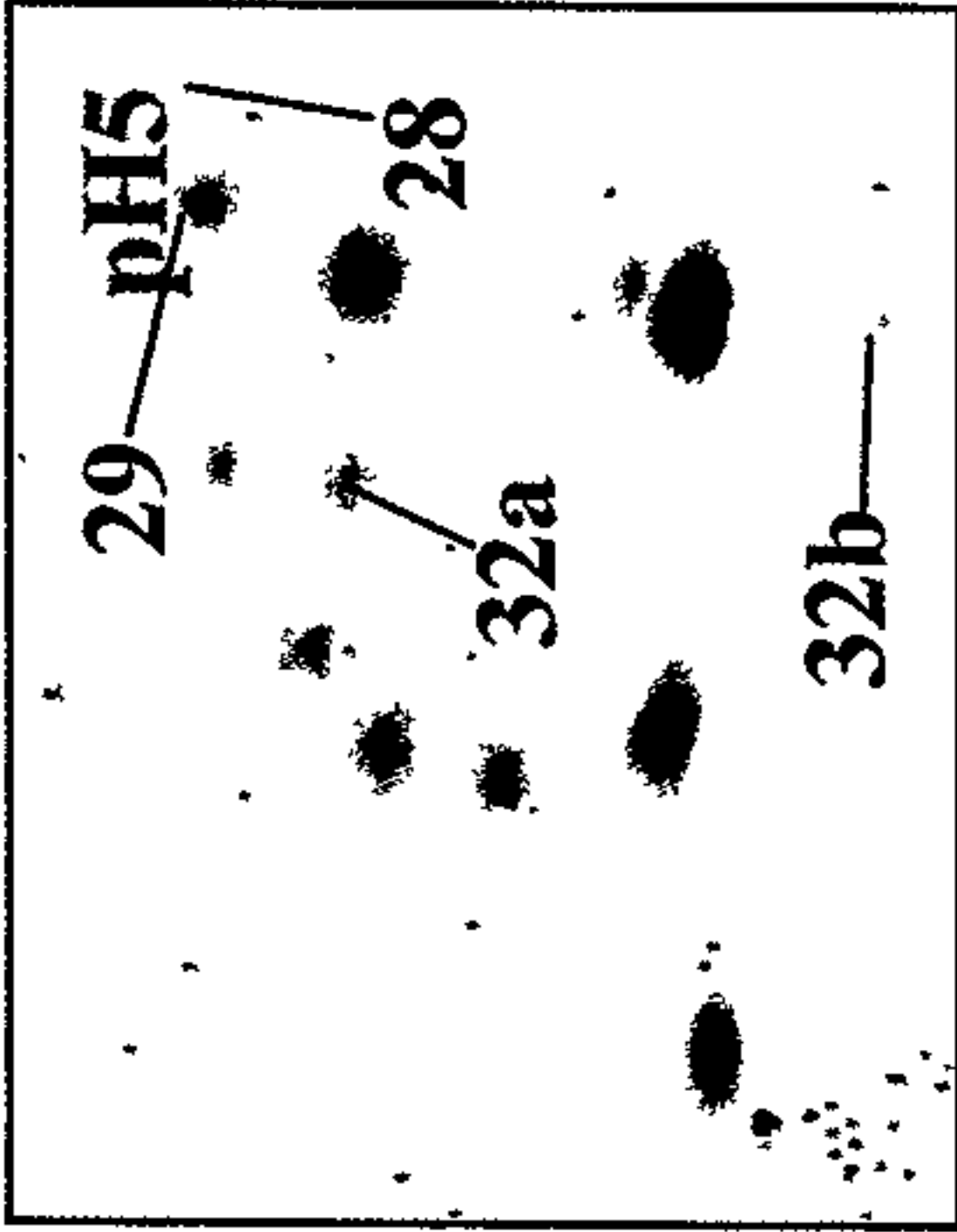
5.7C (cont)

5.7.3

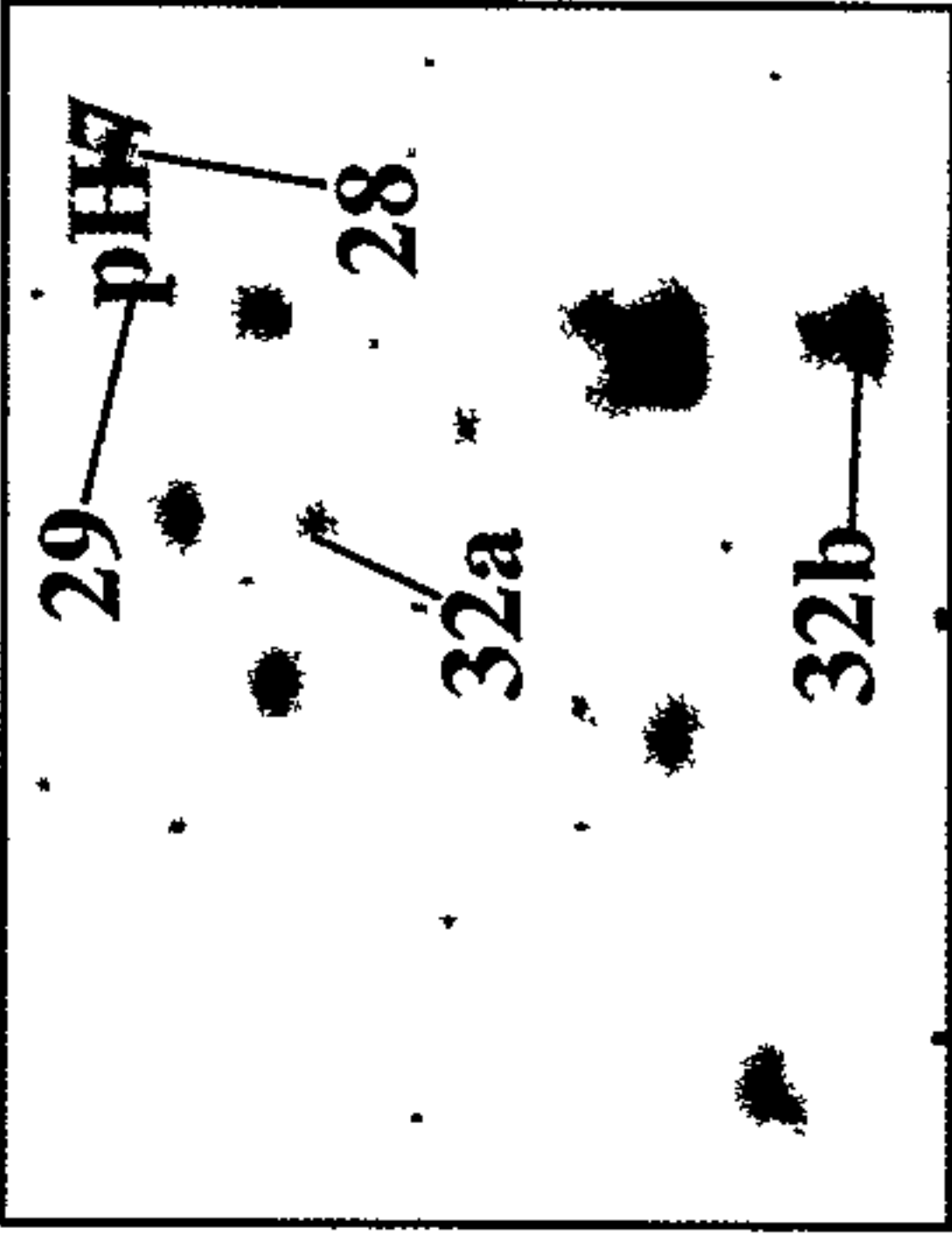
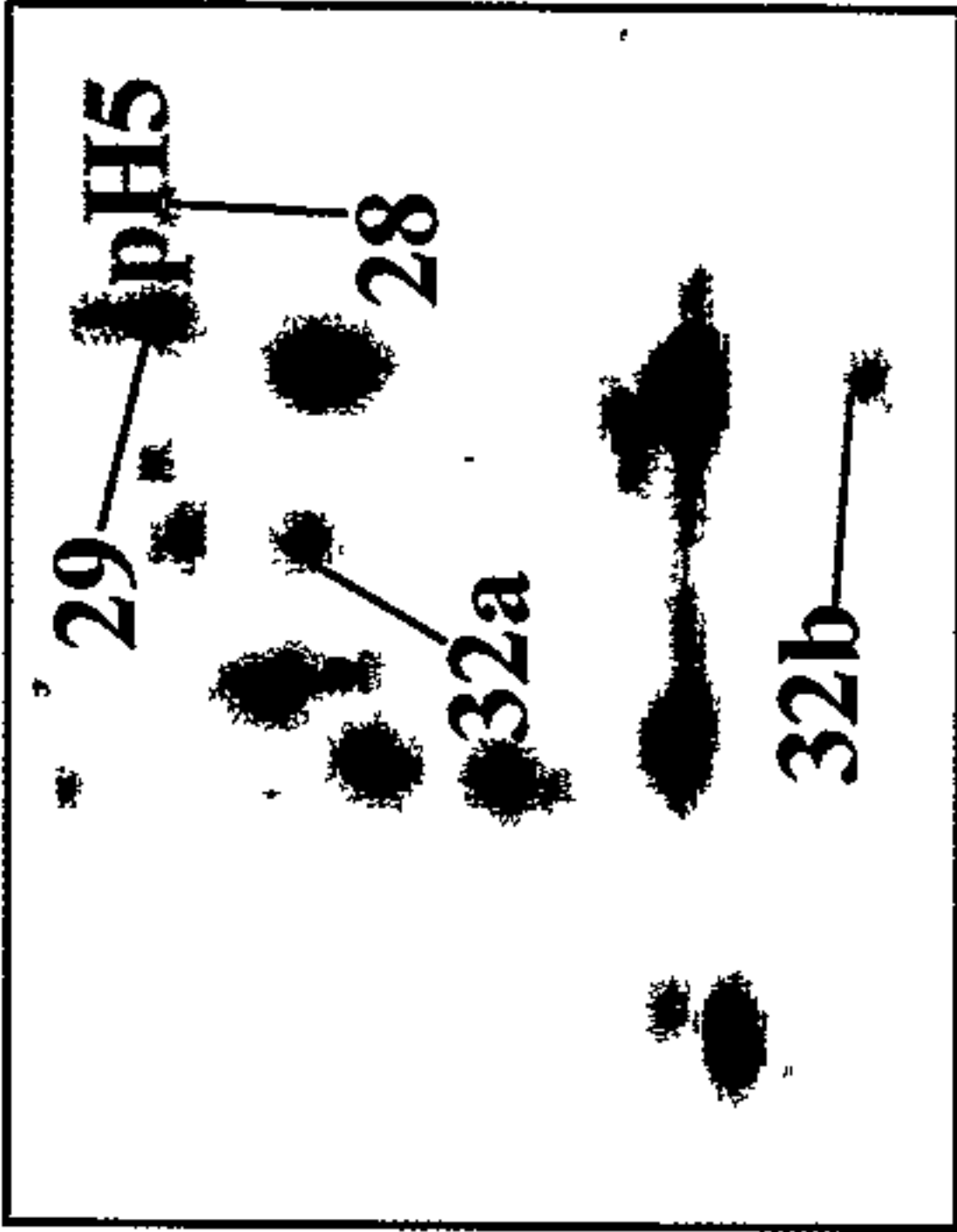
Chemostat 1



Chemostat 2



Chemostat 3



Chapter 6 – General Discussion

A healthy oral cavity maintains a resting plaque pH of ~7.0, while an imbalance towards an acidic pH can result in the initiation of oral diseases such as dental caries. The lowering of the pH in the oral cavity is often observed after the consumption of food, particularly when the food is rich in readily fermentable carbohydrates such as sucrose that are metabolised by saccharolytic oral bacteria. The constant exposure of a tooth to these acidic metabolic end-products promotes the demineralisation of the hydroxyapatite in tooth enamel (Mandel, 1979; Firestone *et al.*, 1982; Rolla, 1989) which in turn may culminate in the irreversible demineralisation of the enamel and the spreading of the disease to the inner tissue components, including the dentine and pulp (Moss, 1998; Billings *et al.*, 2004).

Lowering of the pH in the oral cavity disturbs (in varying degrees) the symbiotic relationship of the resident microflora (Bowden and Hamilton, 1998). Acid intolerant bacteria that are normally associated with healthy dentition include the sanguis group of streptococci (Marsh, 1994; Marsh, 2000; Bowden and Hamilton, 1998). Hamilton and Svensäter (1998) demonstrated that an acid shock from pH 7.5 to pH 5.5 for some of the species that comprise the sanguis group of streptococci elicits a weak response to alteration in protein expression in comparison with the more acid tolerant species such as *S. mutans*. It was suggested that the lack of phenotypic change or more specifically the lack of any increase in proteins required for acid tolerance may have a key role in the inability of these species to tolerate a low pH environment (Hamilton and Svensäter, 1998). This results in the proliferation of other bacterial species that would otherwise have been suppressed at a neutral pH (Svensäter *et al.*, 2003). Prominent bacterial species that thrive under acidic conditions are *Lactobacillus casei* and *Lactobacillus fermentum*, *Actinomyces naeslundii*, *S. sobrinus*, *Streptococcus oralis* and *S. mutans* to name a few (Llena-Puy *et al.*, 2000; Marsh, 2003; Svensäter *et al.*, 2003). Although these bacteria are able to survive at a low pH, not all are always cultivable from a carious lesion. In order to initiate and flourish, these bacteria need to a) adhere to and accumulate on a tooth surface, b) produce water insoluble glucans, c) continue to metabolize at a low pH (be acidogenic), and d) tolerate an acidic milieu (be aciduric) (Burne, 1998). *S. mutans*, in particular, can tolerate and proliferate at a pH as low

as 5.0 and is one of the most frequently isolated bacteria cultured from carious lesions (Tanzer *et al.*, 2001).

S. mutans is a secondary tooth colonizer and a dental plaque resident. *S. mutans* is now recognized as one of the most prominent aetiological agent of dental caries, partly due to its ability to tolerate a low pH environment and continue to metabolize and grow at a low pH (Hahn *et al.*, 1991; Loesche *et al.*, 1975; Loesche *et al.*, 1973; Syed *et al.*, 1975; Minah and Loesche, 1977b; Loesche, 1986; Hamada and Slade, 1980; Takahashi *et al.*, 1997; Sandham and Phillips, 1978). These attributes have led to intensive research into the cariogenic properties (acidogenesis and acidity) of *S. mutans* and its role in dental caries since dental caries remains one of the most significant pandemic diseases of mankind. As such, treatment of dental caries places a significant monetary burden on healthcare systems worldwide.

Many of the earlier studies on dental caries focused on the physiological response of the glycolytic pathway to low pH exposure. Studies included the analysis of the rate of glycolysis at a low pH. In comparison to the rate at a neutral pH (Iwami and Yamada, 1980), changes in the enzyme activity of the major glycolytic enzymes (Takahashi *et al.*, 1997); the rate limiting steps of the pathway (Iwami and Yamada, 1985), and the effects of individual organic acid anions on glycolytic activity were noted (Dashper and Reynolds, 2000). Overall, these studies indicated that acidic conditions decreased the rate of glycolysis and that glucokinase and phosphofructokinase were the rate limiting enzymes. However, prolonged exposure to a low pH did not impair glycolysis in *S. mutans* (Takahashi *et al.*, 1997).

The activity of several glycolytic enzymes in *S. mutans* have been shown to exhibit lower pH optima compared with the corresponding glycolytic enzymes in *S. sanguis* at the same pH (Takahashi *et al.*, 1997). Moreover, it has been found that lactate and formate have a greater inhibitory effect on glycolysis in *S. mutans* than acetate, and that lactate and formate anions decrease glycolytic activity in a concentration dependent manner at pH 5.0. However, the overall sensitivity of *S. mutans* to low pH is less than with *S. sanguis* (Dashper and Reynolds, 2000). That *S. mutans* is less sensitive to acid killing in comparison with other plaque bacteria has been used to explain its ability to proliferate and dominate in dental plaque at a low pH. However,

since much of the focus has been placed on glycolysis, other metabolic pathways and their respective enzymes, which may have been equally as important in facilitating the survival of *S. mutans* at a low pH, were not investigated.

More recent studies that have analysed the effects of pH on *S. mutans* metabolism have utilized both molecular and protein analysis techniques. Differential display reverse transcription-PCR analysis has been used to map the differentially expressed RNA from *S. mutans* using a library of arbitrary 11mer primer designed for *Enterobacteriaceae*. Using an acid-shock model, a protein labelled AP-185 that was found to share 78% homology with branched-chain amino acid aminotransferase was up-regulated when the pH was lowered to pH 5.5 (Chia *et al.*, 2001). In a separate study genetic mutational analysis identified diacylglycerol kinase, an enzyme involved with phosphatidic acid biosynthesis, as necessary for acid adaptation (Yamashita *et al.*, 1993). Another study employed a proteome based approach to investigate the effects of a low pH milieu on *S. mutans* by comparing the protein expression profiles using 2-DGE and MS when *S. mutans* was grown at pH 7.0 and 5.2 in batch culture (Wilkins *et al.*, 2002). This study identified 8 proteins belonging to metabolic pathways other than glycolysis that were differentially expressed by at least 1.5-fold (Wilkins *et al.*, 2002).

S. mutans has been described to elicit an adaptive and genetic response to enable its survival on exposure to an acidic environment, thus allowing it to continue to metabolise and survive (Bowden and Hamilton, 1998; Burne, 1998; Quivey *et al.*, 2000b; Quivey *et al.*, 2001). *S. mutans* acid-tolerance-responses include altering the membrane fatty acid profile (Quivey *et al.*, 2000; Fozo *et al.*, 2004; Fozo and Quivey, 2004), and increasing the expression of the proton-translocating membrane H⁺-ATPase to extrude excess H⁺ (Belli and Marquis, 1991; Bender *et al.*, 1986; Sturr and Marquis, 1992; Hamilton and Buckley, 1991). Although the level of this latter response is catabolite dependent, it can be extended, thus enabling adaptation for prolonged periods of growth at a low pH (Belli and Marquis, 1991; Bender *et al.*, 1986; Sturr and Marquis, 1992).

Mutational studies revealed that DNA repair mechanisms involving RecA and UvrA (Quivey, 1995; Quivey *et al.*, 1995; Hanna *et al.*, 2001), respectively, are essential in maintaining genetic

integrity in *S. mutans* at an acidic pH. In addition to RecA, it was found that an AP-endonuclease, which acts independently of RecA, is also involved in DNA repair (Hahn *et al.*, 1999). Protein structural integrity is also important at a low pH and is maintained by the product encoded by *ffh* (Gutierrez *et al.*, 1999; Kremer *et al.*, 2001; Crowley *et al.*, 2004) as well as the molecular chaperone and misfolded protein degradation enzyme HtrA protease (Diaz-Torres and Russell, 2001). Furthermore, transcriptional analysis also revealed heat shock proteins respond to acid shock (Jayaraman and Burne, 1995; Jayaraman *et al.*, 1997).

It has been determined that it takes 90-120 min to elicit a fully functional ATR (Svensäter *et al.*, 1997). Several studies have been undertaken to analyse the breadth of the protein response using 2-DGE analysis. In one study ¹⁴C-labelled amino acids were incorporated into *S. mutans* cultures grown at pH 7.5 prior to the culture being acid shocked to pH 5.5. Protein profiling demonstrated that 64 proteins were up-regulated at least two-fold. Unfortunately the proteins were not identified (Svensäter *et al.*, 2000). In a second study which compared the 2-DGE proteomes of *S. mutans* grown in batch culture at pH 7.0 and pH 5.2 (Wilkins *et al.*, 2002), 18 proteins were identified by MS to be up-regulated and 12 down-regulated. Surprisingly, DnaK, which had been previously identified as a protein involved with acid-stress-response and subjected to an increase in transcriptional expression (Jayaraman and Burne, 1995; Jayaraman *et al.*, 1997) was found to be down-regulated. Interestingly, the majority of proteins identified to be involved with translation were down-regulated. These apparently inconsistent findings are most likely due to the uncontrolled pH and the significantly dissimilar generation times. At the point of harvest the pH of pH 7.0 and pH 5.2 cultures had fallen to pH 6.2 and pH 4.7, respectively, and the generation times were 1.0 h and 6.6 h at the two pH's, respectively (Wilkins *et al.*, 2002). Both batch cultures were subjected to an ever changing environment making it difficult to determine which aspects of the altered 2-DGE proteome were affected solely by low pH.

These studies have clearly indicated that an acidic environment elicits a change in the expression of *S. mutans* proteins belonging to selective functional groups. However, the model systems used, particularly non-steady state batch cultures, created limitations that affect the interpretation of the results obtained. The model system used in the research reported in this thesis enabled aspects of such limitations to be overcome.

6.1 – Planktonic growth of *S. mutans* using glucose-limited synthetic salivary medium under continuous culture conditions

The experimental aims for this thesis were to first create a reference cellular and extra-cellular proteome map of *S. mutans* grown under glucose-limited homeostatic conditions which would provide the basic parameters necessary for baseline protein expression. This reference map was then employed to compare the effects of a low pH environment on the *S. mutans* proteome profile. Glucose limited-synthetic salivary growth medium was used to support the growth of *S. mutans* and simulate planktonic growth in the oral cavity under resting (in-between meal times) conditions at pH 7.0 and following carbohydrate fermentation (post-dietary carbohydrate intake) where the pH can fall to pH of 5.0. In order to ensure that the differential protein expression was based solely on changes in pH, the bacteria were grown in a chemostat under glucose limited, continuous culture conditions. This method is considered the ‘gold standard’ of cellular cultivation techniques (Lemos *et al.*, 2005), as it provides biological reproducibility through the control of growth parameters thus allowing individual variables of interest to be modified one at a time – in this case the pH of the medium.

6.2 - Exploiting and mapping the *S. mutans* proteome

In order to use a proteome map of *S. mutans* grown at pH 7.0 as the starting reference point for the comprehensive comparison of the changes in the protein expression profile for cells grown at pH 5.0, an optimal protein extraction methodology was required. Several protein extraction methods were evaluated (Chapter 3) before it was found that the combination of lysing cells with mutanolysin and precipitating the proteins using TCA-acetone prior to their solubilisation in MSS was the most optimal for maximizing protein extraction. Initially, broad range IPG strips coupled with SDS-PAGE were employed to separate the proteins. Image analysis of pH 4-7 and pH 6-11 Sypro Ruby stained 2-DGE gels detected 1050 and 200 protein spots, respectively (Fig 3.6). Clearly, the majority of the protein spots were resolved on the pH 4-7 range gels.

Unfortunately, a significant number of proteins demonstrated protein co-migration and overlapping, thereby obscuring low copy number proteins, and potentially hindering the downstream protein identification procedures. These problems were largely overcome by the use of 1 pH unit IPG strips across the pH 4-7 range. A total of 2750 Sypro Ruby stained proteins were detected using narrow range IPG strips thus increasing the number of protein spots detected by 260% in comparison with the equivalent broad range gel (Fig 3.6). From this the first comprehensive proteome map of *S. mutans* was established, which served as a comparative map for *S. mutans* proteins effected by low pH.

6.3 – Protein identification: The availability of genome and protein sequence databases and MS data analysis

The availability of genomic sequences and protein databases on the internet has benefited researchers studying proteomics by enabling the rapid identification and characterisation of proteins. Determining protein identity from a particular species in the absence of a complementary genome sequence database is a major challenge using PMM and sequence data. To partially alleviate the problem, cross species matching can be carried out. However, if the species that is queried is distantly related, the chances of protein identification are slim (Wilkins MR *et al.*, 1997). At the commencement of the experimentation for this thesis (March, 2000) only 100 contiguous DNA sequences greater than 2000 bp of a total of 1,961,176 bp data (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org_search=&org=ntsm02) of *S. mutans* UA159 genome was publicly available. By October 2001, five contiguous DNA sequences were available even though the online protein database such as SWISS-PROT and TrEMBL still only contained around 20 *S. mutans* proteins each. Much of the *S. mutans* PMM data generated from the mapping aspect of this project (Chapter 3) were unable to be identified when the PMM data was used to search SWISS-PROT and TrEMBL. Only *S. mutans* proteins that had been previously identified and entered into the protein databases could be matched along with a few house keeping proteins which share a strong homology across species such as *S. pneumoniae* and *S. pyogenes*. However, this was largely overcome by searching the theoretically-trypsin digested translated protein database from the five contigs of *S. mutans* DNA sequence (section 2.6) using

ProteinLynx software (Micromass) and using the matched protein sequence to query the online databases for homologous proteins. Although post-automated MALDI-TOF/MS provided PMM data for a total of 413 protein spots through automatically picked peak lists, only 50% of this data could be matched using automated peak list with the *S. mutans* translated database. Whilst automated MS and PMM processing is advantageous by virtue of its high-throughput nature, it also poses limitations. Trypsin-digested protein concentrations vary between samples, the way in which they co-crystallise with matrix also varies. Due to this, the laser intensity needed to ionize and release the peptides from the target plate may need adjustment. Moreover, samples spotted on the target plates do not always crystallize homogeneously and if a programmed raster pattern is used for the laser to target the sample on the MALDI plate it can occasionally miss an area on the target sample spot that may contain a concentrated amount or ‘sweet spot’ of the sample. With manual MALDI-TOF/MS analysis, the MS operator is able to choose the target area upon which the laser ionizes the peptides in the sample and change the intensity of the laser accordingly, thereby increasing the chances of finding a ‘sweet spot’ and generating more comprehensive PMM. Manual picking of peak lists from mass spectra can also increase the number of bona fide peptides and minimize contaminants. In this study, 30 % of proteins were identified by manual MS analysis and PMM generation. However the analysis and identification of low copy number proteins and low M_r proteins is still a problem for standard analysis of digested proteins either by automated or manual MALDI-TOF/MS analysis. Micro-concentration of peptides using R2 resin based micro-purification columns (2.9.1) enabled the identification of a number of low copy number proteins resulting in a further 19 % of the proteins being identified. Lastly, proteins that provided good quality mass spectra for PMM but were unable to be matched with the translated *S. mutans* or on-line databases or were derived from low M_r proteins, had to be sequenced using MS/MS prior to identification.

By employing this protein identification strategy, 421 (1% by MS/MS) proteins expressed from 200 ORFs or approximately 11% of those present in the genome of *S. mutans* were identified - the majority being cytoplasmic in nature. This analysis represents the first comprehensive proteome map of *S. mutans* cellular and extracellular proteins. While the majority of the proteins identified from both fractions were cytoplasmic in nature only one integral membrane protein was identified (Table 3.1). Membrane proteins are highly hydrophobic in nature, particularly

intrinsic membrane proteins that have important roles in cell-to-cell signalling and interaction as well as facilitating the movement of molecules across membranes. The identification of only one integral membrane protein was not unexpected given that membrane proteins generally contain hydrophobic moieties which render them highly insoluble (Pasquali *et al.*, 1997; Santoni *et al.*, 2000). The under-representation of intrinsic membrane proteins using 2-DGE based proteome analysis has also been demonstrated in a recent *S. mutans* study (Zuobi-Hasona *et al.*, 2005) and across other bacterial species (Qi *et al.*, 1996; Langen *et al.*, 2000; Molloy *et al.*, 2000; Nilsson *et al.*, 2000; Nouwens *et al.*, 2000; Molloy *et al.*, 2001; Phadke *et al.*, 2001; Cullen *et al.*, 2002; Hommais *et al.*, 2002) and is generally attributed to the fact that the 2-DGE separation system is mainly compatible with easily solubilised proteins (Deshusses *et al.*, 2003). However, this limitation in membrane protein analysis can possibly be overcome by using liquid chromatography based separation of pre-digested proteins to enhance the mapping of the *S. mutans* proteome expressed at pH 7.0 under glucose-limited continuous culture (section 6.5.1) (Washburn *et al.*, 2001; Wu *et al.*, 2003). Such techniques were, however, unavailable during the course of this investigation.

The identification of membrane proteins will be an important future additive to the proteome map of *S. mutans* given that it has been estimated that membrane proteins represent 10-30% of total proteins of bacteria (Wallin and von Heijne, 1998; Santoni *et al.*, 2000), and that this fraction has been under-represented in this study. It is well established that 2-DGE analysis of membrane fractions yields mainly cytoplasmic and peripheral membrane proteins (Qi *et al.*, 1996; Langen *et al.*, 2000; Molloy *et al.*, 2000; Nilsson *et al.*, 2000; Nouwens *et al.*, 2000; Molloy *et al.*, 2001; Phadke *et al.*, 2001; Cullen *et al.*, 2002; Hommais *et al.*, 2002; Zuobi-Hasona *et al.*, 2005), regardless of the effectiveness of the membrane protein isolation protocol. Recently, however, effective membrane protein isolation protocols, such as carbonate-incubation-based membrane protein extraction (Molloy *et al.*, 2000), coupled with multidimensional-protein identification technology (MudPIT)/MS, which combines multidimensional liquid chromatography with ESI-MS, has proven successful in separating and identifying at least 10-fold more *Campylobacter jejuni* integral membrane proteins than 2-DGE/MS based separation and identification protocols (Len *et al.* manuscript in preparation).

MudPIT can also facilitate the detection and identification of low abundance proteins, which are not typically displayed on 2-DGE due to low dynamic range. In addition, low M_r proteins, which either migrate off the bottom of the gel or are difficult to identify based on PMM have an increased chance of detection and identification using MudPIT (Wolters *et al.*, 2001).

6.4 – The effect of a low pH on *S. mutans* protein expression

Global changes in the protein phenotype of *S. mutans* during acid tolerant growth at pH 5.0 have been made possible by using a standardized continuous culture technique, thus enabling a snapshot of the cellular and extracellular proteome during steady state (Chapter 4). This differs from the situation in non-regulated batch cultures, where environmental changes are ongoing throughout growth and during stationary phase. Thus continuous culture in combination with enhanced 2-DGE proteome analysis using both mid-range and narrow-range IPG strips enabled the changes in expression of different isoforms of metabolic proteins to be measured following a transition to an acidic environment. While the additive changes in protein levels clearly impact on the flux of carbon through a given metabolic pathway, it is self-evident that such changes cannot be viewed purely in isolation. Allosteric regulation as well as the stability and relative activity of a given enzyme at a given cytoplasmic pH are important criteria in establishing the ultimate metabolic flux of carbon through a pathway (Yamada and Carlsson, 1975b; Yamada, 1987). While, at one level, the analysis conducted in Chapter 4 has highlighted the extent of phenotypic adaptation during acid tolerance, at another it has shown that changes in the level of expression of proteins appear to be limited to key metabolic pathways; notably, glycolysis, acid production, and the synthesis of branched-chain amino acids. In contrast to previous studies, where none (Svensäter *et al.*, 2000), or fewer than 18 % (Wilkins *et al.*, 2002) of the proteins in these pathways were identified, this study identified 83 % of the enzymes in these three biochemical pathways. The data were consistent with the hypothesis that *S. mutans* adapts to an acidic environment by minimizing H^+ production, using a series of different strategies. This was the case, despite the drain on ATP required for H^+ extrusion and the resultant uncoupling of carbon utilization from anabolic processes (Table 4.2). Nevertheless, Chapter 4 of this thesis has

highlighted the extent to which changes occur in the levels of enzymes involved in central metabolism.

Changes in the levels of protein expression along with measurement of the final yields, Y_{glc} and Y_{ATP} , have substantiated previous findings that streptococci tolerate growth at pH 5.0 by increasing their ability to extrude H^+ while modulating their production of acid fermentation by-products to reduce the level of proton production (Hamilton IR, 1987; Iwami *et al.*, 1992; Miyagi *et al.*, 1994). The results further suggest that *S. mutans* utilizes branched chain amino acid biosynthesis to lower the amount of reducing equivalents in the form of NAD(P)H that would otherwise be converted to acidic by-products and by so doing help buffer the cytoplasm by producing NH_3^+ . The exact role of branched chain amino acid biosynthesis in acid resistance in *S. mutans* and the validity or otherwise of this hypothesis is currently the subject of further investigation in our laboratory (section 6.6, Appendix 2). The observed alterations in the levels of expression of proteins, however, clearly reflect the complexity required to maintain a phenotype commensurate with the survival of *S. mutans* in an acidic environment. In this regard, the nature of the multiple protein forms (particularly isoforms) remains to be elucidated, as do the mechanism(s) by which such an integrated change in the level of proteins is coordinated.

The proteome literature relating to acid adaptation and tolerance in *S. mutans* contains disparate findings. For instance, proteome analysis of *S. mutans* during acid adaptation, using an aerobic batch-culture model without pH control (Wilkins *et al.*, 2002), identified eight of the 28 differentially expressed species of stress protein reported in Chapter 5. The levels of six of these were down-regulated by an average of 2.6-fold, while DNA-directed RNA polymerase was down-regulated 33-fold. Only a 60-kDa chaperonin was up-regulated to a similar extent to that observed with GroEL (Chapter 5). It is difficult to assess whether the aerobic conditions, the use of a different culture medium and/or the dissimilar generation times of 1.0 h and 6.6 h, respectively, for the control and the experimental batch cultures influenced the outcome (Wilkins *et al.*, 2002). Since an arbitrary ratio of the levels of expression has been evaluated in both studies, it may be that, in batch culture, the control bacteria are adapting to a fall in the extracellular pH from a starting value of pH 7.0 to pH 6.2 at harvest (heading for a final of pH 5.3 at stationary phase) by initially over expressing stress-related proteins. This would contrast with the steady-state levels measured in a chemostat once *S. mutans* had adapted to the prevailing pH conditions. Such an overshoot in enzyme levels as an initial response to change has

been noted previously, albeit in continuous culture (Carlsson and Elander, 1973; Koplove and Cooney, 1978), and if occurring in batch culture would explain the apparent reduction in the ratio between control (starting pH 7.0) and experimental (starting pH 5.2) protein values observed. Whatever the reason for the disparity, one must conclude that the two models in fact reflect dissimilar events.

Although a number of stress-related proteins in both Gram-positive and Gram-negative bacteria are either well characterized or are the subject of concerted ongoing study, others are not. The results presented in Chapter 5 have described the involvement of at least five novel proteins in the acid-tolerant growth of *S. mutans*. These include the transcription elongation factor, GreA; the ATPase protease, ClpL; and the single-stranded DNA-binding protein, Ssb; polyribonucleotide nucleotidyl transferase, PnpA; and a protein having homology to *L. helveticus* dipeptidase, PepD.

6.5 – Recent developments in proteomic analysis

In this study Sypro Ruby stained 2-DGE gels were used for comparative proteome analysis using z3 image analysis software. This method was deemed the best and most appropriate method available for obtaining quantitative and qualitative proteome data. Methods such as fluorescence 2-D difference gel electrophoresis (DIGE) technology which allows multiplexing of different samples on one 2-DGE gel were still in their infancy. DIGE has now superseded traditional 2-DGE based comparative methods as it significantly reduces gel-to-gel variation and laborious bench time needed to run 2-DGE replicate gels (Alban *et al.*, 2003; Karp *et al.*, 2005). Essentially, DIGE allows for the simultaneous analysis of up to three samples; internal standard, control, and the sample of interest each labelled with spectrally distinct and resolvable CyDyes (Cy2, Cy3 and Cy5 [GE Healthcare, Uppsala, Sweden]) prior to multiplexing on a single 2-D gel. DIGE can be employed to validate findings as well as potentially provide more information on differentially expressed proteins. The advantage of the application of DIGE for comparative studies of changes in the phenotype of *S. mutans* are readily exemplified by recent studies in our laboratory which have compared data obtained using the traditional 2-DGE approaches used in the studies reported in this thesis and DIGE analysis. Statistically significant changes that could

only be surmised by traditional methods were confirmed using DIGE (Rathsam *et al.*, 2005a; Rathsam *et al.*, 2005b).

Whilst MudPIT can provide invaluable qualitative data it is limited in that quantitative data can not be generated by this technology alone. However, MudPIT coupled with iTRAQ labelling technology which can analyse up to four samples simultaneously (Shadforth *et al.*, 2005), can potentially overcome this limitation. In a recent study investigating the membrane sub-proteome of the Gram positive bacterium *Deinococcus radioduran* it was reported that significantly more hydrophobic membrane proteins were identified when labelled with iTRAQ in comparison with those labelled with iCAT (Blonder *et al.*, 2002; Goshe *et al.*, 2003). The future application of this coupled labelling and separation technology will clearly expand our understanding of differentially expressed proteins in *S. mutans* grown under different environmental conditions including low pH.

6.6 - Investigating the roles of genes expressing products that demonstrate differential or unique expression at pH 5.0.

Several pathways and proteins exhibited up-regulated or unique expression at pH 5.0. Of particular interest is the up-regulation of two key enzymes involved with the branched chained amino acid biosynthetic pathway (section 4.3.7). Interestingly, analysis of *S. mutans* codon usage shows that 24.4% of the amino acids of its proteome are branched-chain amino acids, indicating there is a high biosynthetic demand for these amino acids. The creation of mutants with either a mutation in *ilvC* and *ilvD* gene (Appendix 2) should allow one to elucidate the physiological significance of the products of these genes in the proliferation and survival of *S. mutans* in a low pH environment. Similar molecular studies using isogenic mutants can be used to investigate other genes implicated in acid tolerant growth of *S. mutans* at low pH, for example, genes involved with stress response (section 5.4).

6.7 – Conclusions

Despite the fact that the molecular response of *S. mutans* to acid exposure, such as that experienced in carious plaque, is complex, the availability of technologies such as proteomics coupled with a complete genome sequence makes it possible to take a global approach to investigating protein expression. The goals of this study were to use proteomics to analyse global protein expression in *S. mutans* by mapping its phenotype under baseline conditions and then subsequently comparing this phenotype with that expressed at a low pH. By cultivating *S. mutans* at steady state under glucose-limited continuous culture conditions at pH 7.0 or pH 5.0 in a chemostat with a dilution rate of 0.1 h^{-1} , it was possible to establish comprehensive 2-DGE gel maps for each pH. The pH 7.0 map provided a baseline for comparison with the pH 5.0 map making it possible to understand the effect of a low pH environment on global protein expression in *S. mutans*. The data obtained from these studies showed that the expression of enzymes involved with central metabolism as well as DNA and protein repair were altered in their levels of expression to facilitate the adaptation and continued survival of *S. mutans* during acid tolerant growth at pH 5.0.

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APPENDIX 1 – Media used to support *S. mutans* and *E. coli* growth

Brain Heart Infusion(BHI) broth

3.7% (w/v) Brain Heart Infusion

The media was autoclaved at 15 psi for 15 min.

BHI broth (solid media)

3.7% (w/v) Brain Heart Infusion

1.5% (w/v) bacteriological agar

The media was autoclaved at 15 psi for 15 min.

Horse Blood Agar (HBA)

Horse blood agar constitutes Brain Heart Infusion broth (solid media) with the addition of 5.0% (v/v) Defibrinated horse blood (50 ml L⁻¹) following autoclaving.

***Luria-Bertani (LB) broth* (Sambrook *et al.*, 1989)**

1 % (w/v) bacto-tryptone

0.5 % (w/v) bacto-yeast extract

1% (w/v) NaCl

The pH of the media was adjusted to pH 7.0 using 5M NaOH and then autoclaved at 15 psi for 15 min

DMM medium (Sissons *et al.*, 1991)

The following components were made prior to mixing together with other DMM medium components:

(i) Amino acid cocktail

Alanine	1.74 g
Arginine.HCl	2.74 g
Aspartic acid	2.02 g
Glutamic acid	7.96 g
Glycine	1.46 g
Histidine	1.68 g
Isoleucine	3.12 g
Leucine	4.83 g
Lysine.HCl	5.53 g
Methionine	1.62 g
Phenylalanine	2.86 g
Proline	4.24 g
Serine	3.64 g
Threonine	1.29 g
Tyrosine	3.92 g
Valine	2.79 g
<u>18 MΩ H₂O</u>	<u>948.56 mL</u>
Final volume	1000 mL

To effectively solubilise the amino acids the solution was stirred over heat.

(ii) Adenine and Guanine solution

Adenine	200 mg
Guanine	200 mg
<u>18 MΩ H₂O</u>	<u>99.6 mL</u>
Final volume	100 mL

Concentrated HCl to effect solution.

(iii) Salivary amino acids

Alanine	4.45 g
Arginine HCl	10.53 g
Asparagine	3.75 g
Aspartic acid	3.33 g
Cysteine HCl,	7.88 g
Glutamic acid	3.68 g
Glutamine	3.65 g
Glycine	7.10 g
Histidine	1.55 g
Isoleucine	3.28 g
Leucine	3.28 g
Lysine HCl	9.13 g
Methionine	1.49 g
Phenylalanine	4.13 g
Proline	11.51 g
Serine	2.63 g
Taurine	9.38 g
Threonine	2.98 g
Tryptophan	2.04 g
Tyrosine	2.72 g
Valine	2.93 g
18 MΩ H ₂ O	896.66 mL
Final volume	1000 mL

(iv) Vitamin cocktail

Biotin	24 mg
Creatinine. HCl	149 mg
Cyanocobalamin	68 mg
Folic acid	11 mg
Menadione	1381 mg
Niacin	616 mg
PABA	137 mg
Pantothenic acid	239 mg
Pyridoxine.HCl	822 mg
Riboflavin	113 mg
Thiamine.HCl	337 mg
18 MΩ H ₂ O	996.1 mL
Final volume	1000 mL

The following components were mixed together before autoclaving for 10 L of media:

Amino acid cocktail (see above)	1.00 L
Uric acid	84.05 g
Adenine and Guanine solution (see above)	0.10 L
Uracil 0.200% (w/v)	0.01 L
Choline chloride 1.396 % (w/v)	0.01 L
Sodium citrate 1.470 % (w/v)	0.01 L
Haemin 0.652 % (w/v)	0.01 L
(NaOH to effect solution)	
MgCl ₂ .6H ₂ O	0.41 g
KH ₂ PO ₄	20.05 g
K ₂ HPO ₄ .3 H ₂ O	34.00 g
NaCl	5.84 g
KCl	11.18 g
NH ₄ Cl	1.07 g
<u>18 MΩ H₂O</u>	<u>8.50 L</u>
Final volume	9.80 L

The pH of the final mixed solution was adjusted to 6.8, autoclaved at 15 psi for 30 min and cooled before the addition of post autoclaving additives (below).

10 mL of sterile CaCl₂.2H₂O 14.7% (w/v) was added aseptically and separately to the autoclaved component of the media.

The following components were made up fresh:

Salivary amino acids (see above)	0.01 L
Tryptophan	0.880 g

Stir the above two components in 190 mL of 18 MΩ H₂O over heat until both components are completely dissolved then allow to cool to 16 °C. Once solution has cooled add the following components:

Urea	0.600 g
Asparagine	2.600 g
Cysteine.HCl	0.079 g
Glutamine	4.430 g
Ascorbic acid	0.009 g
NaHCO ₃	4.200 g
Glucose	45.000 g
Vitamin cocktail (see above)	0.01 L

The final mixed solution was filter sterilized using a Minisart, syringe filter (filter pore size: 0.20 μM) (Sartorius) and added to the above mentioned autoclaved component of the media aseptically.

APPENDIX 2 – The insertional inactivation of genes, *ilvC* and *ilvD*, involved in branched-chain amino acid biosynthesis

The comparison of 2-DGE proteome profiles of *S. mutans* cells grown at a steady state in a chemostat under glucose limitation at pH 7.0 and pH 5.0 revealed several proteins belonging to the branched-chain amino acid biosynthetic pathway that were up-regulated at pH 5.0. To investigate the role in acid adaptation of the genes *ilvC* and *ilvD* a molecular approach was taken by creating individual mutants with a mutation in each of these genes as will be described below.

Bacterial strains and growth conditions. Bacterial strains and plasmids that were used are listed in Table 1. Permanent stocks of all strains were maintained at -80°C in 25% (v/v) glycerol with the appropriate growth media described below. *S. mutans* was cultivated without agitation under anaerobic conditions (95% N₂, 5% CO₂) at 37°C in BHI broth (Appendix 1) supplemented with 0.3% (w/v) yeast extract (BHIY). *S. mutans* LT11*ilvC* and *S. mutans* LT11*ilvD* mutants (Table 1), were selected on BHIY broth supplemented with 15 µg erythromycin mL⁻¹. *E. coli* was grown under aerobic conditions with agitation at 37°C in LB broth (Appendix 1) supplemented with 100 µg ampicillin mL⁻¹.

Primer design

Oligonucleotides were designed to amplify a region 100 base pairs downstream of the start codon of the genes of interest. This was carried out in order to avoid amplifying the start of the 5' region from either gene that may encode for a signal peptide. A prerequisite for the primers was that they contained at least 20 bases and a T_m of ≥55°C. These criteria were checked using an 'oligo calculator' (<http://micro.nwfsc.noaa.gov/protocols/oligotmcalc.html>). The primers for *ilvC* were; 5'-GAGAGATTCAGGACACGATGTTATC-3' (forward) and 5'-TTACAAGGAGACCTACACGC GC -3' (reverse) and are complementary to positions 113-135 and 544-523, respectively. For *ilvD* the oligonucleotides primers were 5'- GCTATGTTGCGTGCAACTGG -3' (forward) and

5'- GTGTTAGCAGTATACATACCACCG -3' (reverse) and are complementary to positions 79-98 and 603-626, respectively. Primers were synthesized by Proligo (Sigma).

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference and/or source
<i>E. coli</i>		
XL1-Blue MRF'	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)].	Stratagene
XL1-Blue MRF' <i>ilvC</i>	pALA030	This study
XL1-Blue MRF' <i>ilvD</i>	pALA050	This study
<i>S. mutans</i>		
LT11		(Tao <i>et al.</i> , 1993)
LT11 <i>ilvC</i>	<i>ilvC</i> ::pVA8912 Erm ^R	This study
LT11 <i>ilvD</i>	<i>ilvD</i> ::pVA8912 Erm ^R	This study
Vectors		
pGEM-T Easy	Amp ^R , 3' T overhangs	Promega
pVA8912	pVA8911 without 412-bp PvuII fragment, Erm ^R	(Malke <i>et al.</i> , 1994)
Plasmids		
pALA030	434 bp amplicon homologous to the 5' <i>ilvC</i> Amp ^R	This study
pALA031	<i>Pst</i> I- <i>Sph</i> I restricted from pALA030 containing <i>ilvC</i> amplicon; Erm ^R	This study
pALC050	548 bp amplicon homologous to the 5' <i>ilvD</i> Amp ^R	This study
pALC051	<i>Pst</i> I- <i>Sph</i> I restricted from pALA050 containing <i>ilvD</i> amplicon; Erm ^R	This study

PCR amplification and plasmid construction.

PCR amplification

A 100 μL PCR reaction mix containing 18 MΩ H₂O, 50 μL HotStarTaq Master mix (200 μM of each deoxynucleotide triphosphate (dNTP)¹³, 2.5 units HotStarTaq DNA polymerase, 1.5 mM MgCl₂ [Qiagen, Hilden, Germany]), 0.1 μM of appropriate forward and reverse primers (above), 100 ng *S. mutans* LT11 genomic DNA was used for PCR amplification of either the *ilvC* or *ilvD*

¹³ dNTPs consist of: 2'-deoxyadenosine 5'-triphosphate (dATP), 3'-deoxythymidine 5'-triphosphate (dTTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxycytidine 5'-triphosphate (dCTP).

gene. PCR amplification was carried out using a FTS-320 Thermal Sequencer (Corbett Research, Sydney, Australia) with one cycle of initial denaturation followed by 35 cycles of thermocycling and one cycle for final extension (Table 2).

Amplified regions of *ilvC* and *ilvD* were separated by 1% (w/v) agarose gel electrophoresis and the amplified sequences excised and gel purified using QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions. Electrophoresis of DNA was carried out by using electrophoretic grade agarose, 1% (w/v), which was dissolved in 1 x Tris-acetate-EDTA (TAE) electrophoresis buffer made from 50 x stock solution pH 8.5 (24.2% (w/v) Tris base, 5.71% (v/v) glacial acetic acid, 3.72% (w/v) Na₂EDTA.2H₂O in 18 MΩ H₂O) by heating in the microwave with occasional stirring. The gel was allowed to cool to approximately 60°C before being supplemented with 2 µL of 10mM ethidium bromide, mixed thoroughly, and poured into casting trays to set. DNA samples were mixed with loading dyes (Bio-Rad) according to the manufacturer's instructions and electrophoresed at 100 V in a Mini-Sub Cell GT Electrophoresis Cell (Bio-Rad). DNA-ethidium bromide complexes were viewed under a UV 302 nm transilluminator.

Recombinant plasmids pALA030 and pALA050 were constructed by ligating the amplified regions of the *ilvC* and *ilvD* genes, respectively, into pGEM-T Easy (Promega, Madison, WI, USA) (Table 1). This was achieved in a 10 µL ligation reaction in 18 MΩ H₂O containing 100 ng of pGEM-T Easy, 33 ng amplicons of either *ilvC* or *ilvD*, 2x ligation buffer (Promega) and 3 Weiss units of T4 DNA ligase (Promega) at 4°C for 16 h.

Table 2. Thermocycling parameters

Stage	Temp.(°C)	Time(min)
Initial denaturation	95	5
Thermocycling	95 53 72	1 1 1
Final extension	72	15

Transformation of *Escherichia coli* XL1-Blue MRF'.

Recombinant plasmids were transformed into *E. coli* XL1-Blue MRF' (Stratagene) (Table 1) by electroporation using a Gene Pulser™ (BioRad; 200 Ω m, 2.45 V, 25 μ F, $\sim$ 4.6 sec). Transformed cells were incubated on ice for 1 min and 450 μ L of pre-warmed LB broth then added and the suspended cells incubated for a further 50 min at 37°C. Transformants were then plated onto LB agar containing 100 μ g ampicillin mL⁻¹ agar plates supplemented with 100 μ L of 20 mg isopropyl-beta-D-thiogalactopyranoside (IPTG) mL⁻¹ and 40 μ L of 20 mg of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) mL⁻¹ and subsequently incubated at 37°C for 16 h. Colonies containing recombinant plasmids were screened according to blue-white selection and white colonies were picked and cultivated with agitation at 37°C for 16 h in LB broth containing 100 μ g ampicillin mL⁻¹. Plasmids were subsequently extracted from the *E. coli* cells using Wizard®*Plus* SV Minipreps DNA purification system (Promega) according to the manufacturer's instructions. To confirm the nature of positive clones, amplicons containing partial *ilvC* and *ilvD* sequences were excised from purified recombinant vectors pALA0030 and pALA0050 (Table 1) using the restriction enzymes *Pst*I (Promega) and *Sph*I (Promega), according to manufacturer's instructions and separated on 1% (w/v) agarose as described above (Fig. 1). Amplicon DNA sequencing was performed using an Applied Biosystems Model 373A DNA sequencer using 'Big-Dye' terminator (version 3) chemistry by the DNA Sequencing Facility, Westmead Millennium Institute, Westmead, Australia.

Cloning of partial sequences *ilvC* and *ilvD* into pVA8912.

Following the separation of restricted recombinant vectors pALA0030 and pALA0050 by 1% (w/v) agarose gels, double *Sph*I and *Pst*I restricted amplicons were excised from the gel and subsequently purified using QIAquick gel extraction kit (Qiagen). Purified restricted amplicons were quantified according to manufacturer's instructions using a PicoGreen® dsDNA quantitation kit (Molecular Probes) and then ligated into the vector pVA8912 (Table 1; Malke *et al.*, 1994), which had been cleaved with *Pst*I and *Sph*I as described above for pGEM-T Easy.

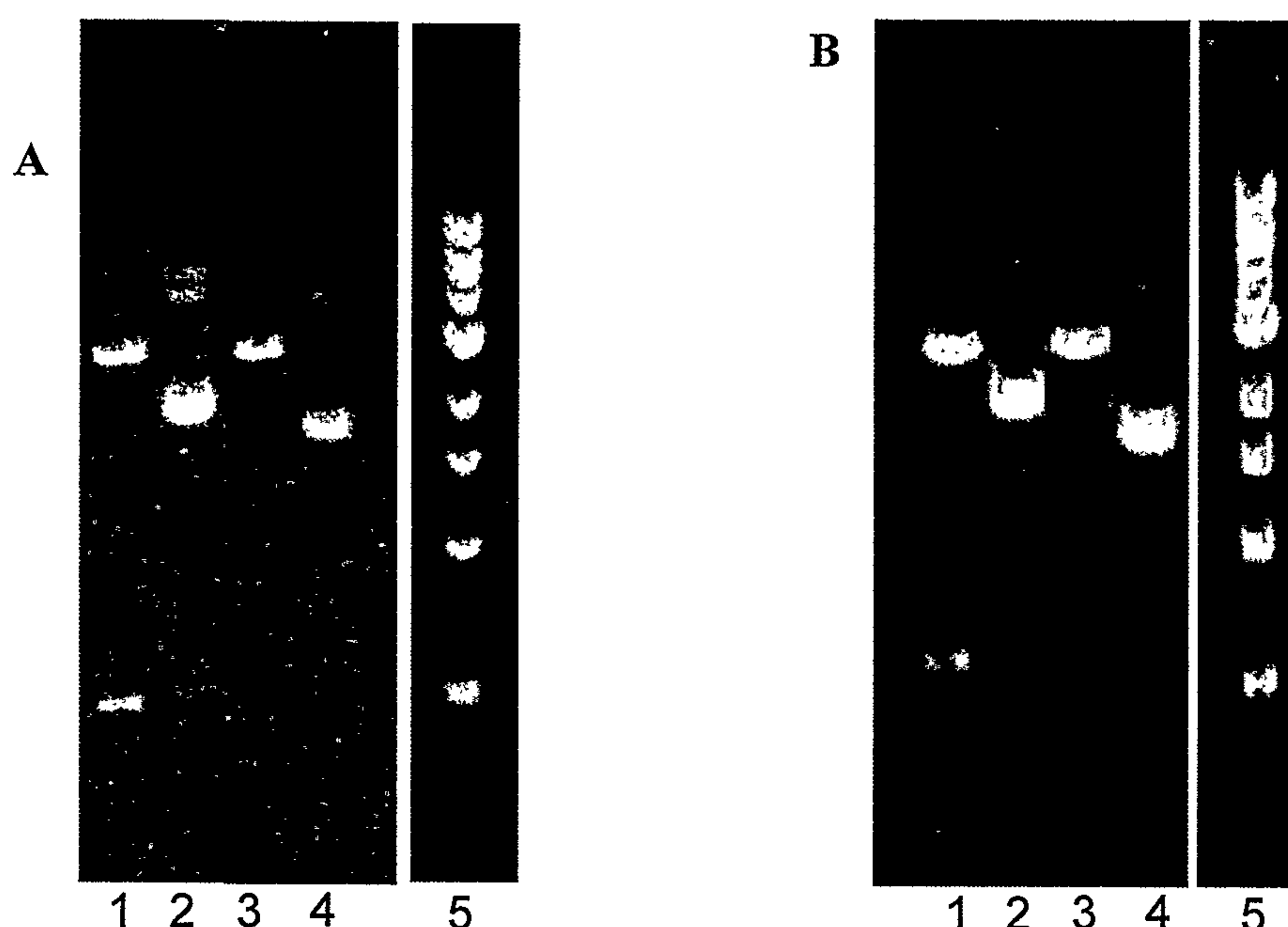


Figure 1. Gel electrophoresis of restricted digests of pALA0030 and pALA0050. Lane 1, *SphI-PstI* double digest of pALA0030 (A) and pALA0050 (B); lane 2, pALA0030 (A) and pALA0050 (B); lane 3 *SphI-PstI* Double digest pGEM-T Easy (A and B); lane 4, pGEM-T Easy (A and B); lane 5, 1000 bp marker (A and B).

The recombinant plasmids were then individually transformed into *E. coli* XL1-Blue MRF' using the transformation protocol (described above). Screening, propagation of positive colonies, plasmid extraction and confirmation of positive clones by sequencing, were all performed as described above except that 15 μg erythromycin mL^{-1} was used where appropriate (Fig. 2).

Construction of *ilvC* and *ilvD* mutants.

Purified pALC031 and pALC051 (Table 1) were individually transformed into *S. mutans* LT11 using a modified protocol of the method described by Tao *et al.* (1993). Briefly, 10 mL of pre-heated (37°C) BHIY was inoculated with 250 μL (1:40) of an *S. mutans* LT11 overnight culture (16 h) and incubated at 37°C for 2.5 h with shaking. A 1 mL aliquot of the growing culture was then transformed with 250-400 ng of either pALC031 or pALC051 for 4 h at 37°C. Following transformation, 100 μL aliquots of transformed cells were plated onto BHI agar plates (Appendix 1) supplemented with 15 μg erythromycin mL^{-1} and incubated at 37°C for 48 h.

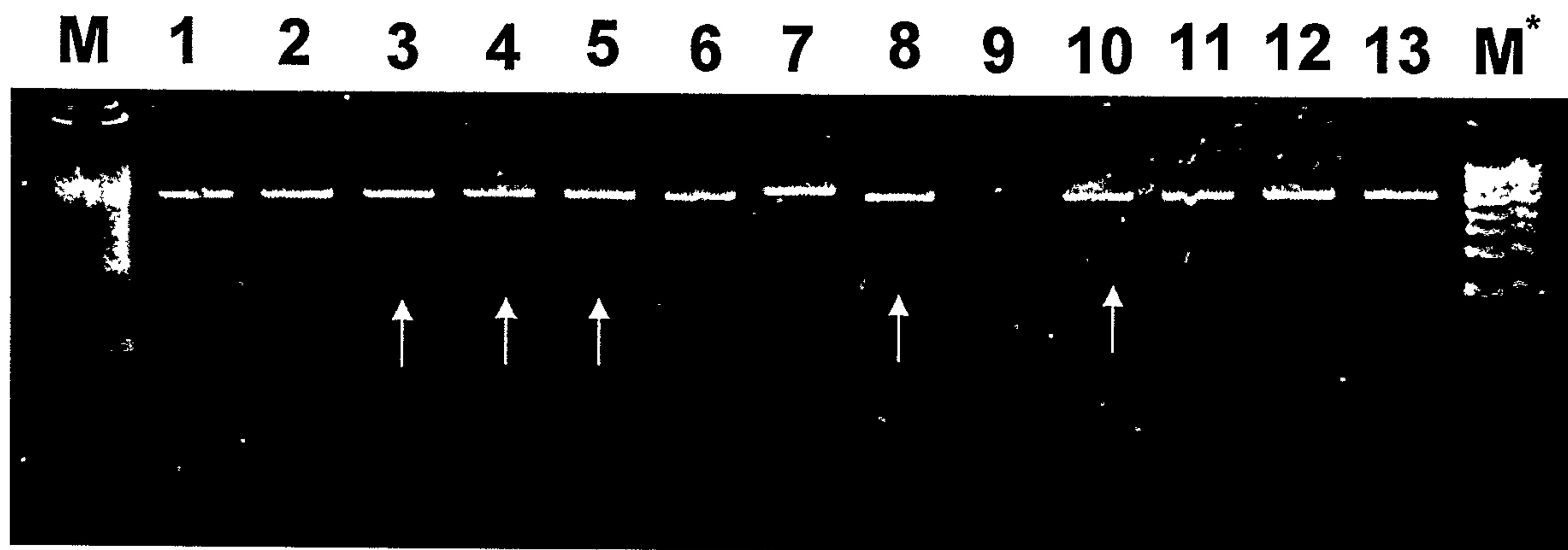


Figure 2. Confirmation of positive *ilvC*::pVA8912 (lanes 1-8) and *ilvD*::pVA8912 (lanes 10-13) using *SphI*-*PstI* double digestion; positive *ilvC*::pVA8912 (arrow, lanes 3-5 and 8); positive *ilvD*::pVA8912 (arrow, lane 10); 100 bp marker (lane M [Promega]); 1000 bp marker (lane M* [Promega]).

Confirmation of *ilvC* and *ilvD* mutants by Southern blotting.

DNA isolation

Genomic DNA from both *S. mutans* LT11 *ilvC* and *ilvD* mutants were extracted using a modification of the methods described by Giffard *et al* (1991). The mutants were grown in 10 mL of BHIY supplemented with 10 mM DL-threonine (BHIYT) as described above. A further 10 mL of BHIYT was then added to the cultures which were grown to late exponential phase (OD_{600} 0.6-0.8), at which point 5% (w/v) glycine was added to the culture and incubation continued for a further 1.5-2.0 h under the same growth conditions. The cells were then harvested by centrifugation (10 000 $\times$ g, 4 $^{\circ}$ C, 15 min), the supernatant discarded and the pellet resuspended in 1 mL of 10mM Tris-HCl pH 8.0 containing 1mM EDTA (TE) buffer. The resuspended cells were then centrifuged (10 000 $\times$ g, 4 $^{\circ}$ C, 1 min), the supernatant discarded and the pellet resuspended in 600 μ L of TE. The resuspended cells were supplemented with 0.2% (w/v) lysozyme (Roche, Basel, Switzerland), 1 μ L of 10mg RNaseA mL⁻¹ (Sigma), 15 μ L of 1 mg mutanolysin mL⁻¹, and 10 μ L of 10mg Proteinase K mL⁻¹ (Sigma) and incubated at 50 $^{\circ}$ C for 3h. An aliquot of 1% (w/v) SDS was then added and the samples incubated for another 10 min.

The sample was then diluted to final volume of 1.5 mL with TE, mixed gently by inversion and incubated at 50°C for 10 min. The resulting translucent viscous solution was extracted 4x with an equal volume of Tris/HCl-saturated phenol pH 8.0 and once with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). Following extraction, DNA was precipitated using a 0.1 volume of 3M sodium acetate pH 5.2 and 2.5 volumes of ice cold ethanol. Subsequently DNA was spooled onto a glass Pasteur pipette, washed in 70% (v/v) ethanol and then dissolved in 500 µL of TE.

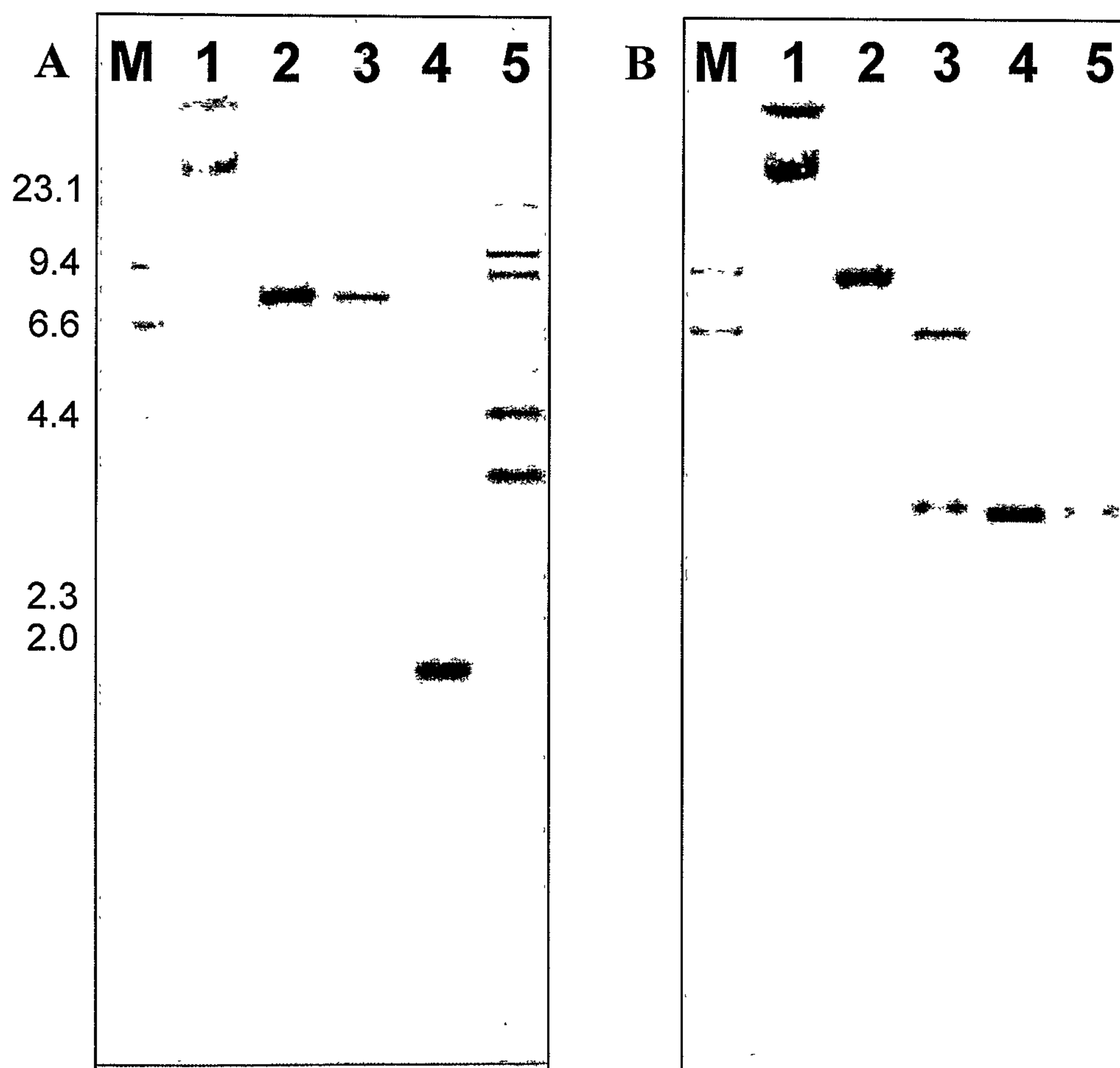
Southern Blot

Mutants were confirmed by Southern hybridisation using PCR digoxigenin labelled probes. Isolated chromosomal DNA from the *S. mutans* LT11 *ilvC* and *ilvD* mutants were digested with *EcoRI* and *HindIII* and separated on 1% (w/v) agarose as described above. Southern transfer of DNA was performed according to Southern blotting protocol described in 'The DIG System user's guide for filter hybridisation' (Boehringer Mannheim [Roche, Basel, Switzerland]). *IlvC* and *ilvD* amplicons were produced by incorporating of Digoxigenin-11-dUTP during PCR amplification using DIG DNA labeling kit (Boehringer Mannheim [Roche]) following the manufacturer's instructions. These were used as probes for hybridization in the Southern blots. Colorimetric detection of hybridized probes made use of the detection reagents nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl-phosphate according to the manufacturer's instructions (Fig. 3).

Use of mutant strains

These mutants are available to the laboratory for future use in the analysis of the physiological significance of *ilvC* or *ilvD* mutation in *S. mutans* LT11 when grown under steady state in a glucose limited chemostat at pH 7.0 or pH 5.0.

Figure 3. Southern Blot analysis of *S. mutans* LT11 *ilvC* (A) and *ilvD* (B). Lane 1 *S. mutans* LT11 *ilvC*⁻ chromosomal DNA (A), lane 2 *Eco*RI digest of *S. mutans* LT11 chromosomal DNA (A), lane 3 *Eco*RI digest of *S. mutans* LT11 *ilvC*⁻ chromosomal DNA (A), lane 4 *Hind*III digest of *S. mutans* LT11 chromosomal DNA (A), lane 5 *Hind*III digest of *S. mutans* LT11 *ilvC*⁻ chromosomal DNA. Lane 1 *S. mutans* LT11 *ilvD*⁻ chromosomal DNA (B), lane 2 *Eco*RI digest of *S. mutans* LT11 chromosomal DNA (B), lane 3 *Eco*RI digest of *S. mutans* LT11 *ilvD*⁻ chromosomal DNA (B), lane 4 *Hind*III digest of *S. mutans* LT11 chromosomal DNA (B), lane 5 *Hind*III digest of *S. mutans* LT11 *ilvD*⁻ chromosomal DNA.



APPENDIX 3 - PUBLICATIONS

Len ACL, Cordwell SJ, Harty DWS, Jacques NA. 2003. Cellular and extracellular proteomic analysis of *Streptococcus mutans* grown in a chemostat. *Proteomics* 3: 627-646.

Len ACL, DWS Harty, NA Jacques. 2004. Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid stress. *Microbiology* 150: 1353-1366.

Len ACL, DWS Harty, NA Jacques. 2004. Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. *Microbiology* 150: 1339-1351.

Rathsam C, RE Eaton, Len ACL, NA Jacques. 2005. A proteomic approach to understanding the biofilm phenotype. *Australia Microbiology*. 26 (3): 120-121.

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Cellular and extracellular proteome analysis of *Streptococcus mutans* grown in a chemostat

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The oral pathogen, *Streptococcus mutans*, was grown under glucose limitation in a chemostat at pH 7.0 and a dilution rate of 0.1 h⁻¹ to mimic the conditions prevailing in a healthy human oral cavity in between meal times. Solubilized cellular and extracellular proteins were separated by two-dimensional gel electrophoresis (2-DE) and, following tryptic digestion, 421 protein spots analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry or electrospray ionization-tandem mass spectrometry. Analyses of the mass spectral data showed that the proteins matched the translation products of 200 different open reading frames (ORFs) deduced from contigs of the *S. mutans* UA159 genome and thus represented proteins derived from approximately 11% of the total ORFs of the bacterium. Of the identified proteins, 172 (including one surface protein) were characterized in the cellular fraction, and the remaining 28 (including two surface proteins) were uniquely identified from the culture fluid. The expression and therefore the existence of 30 proteins previously designated as 'hypothetical' or with no known function was confirmed. 2-DE of whole cell lysates revealed only a single intrinsic membrane protein. This is consistent with proteomic analyses of other Gram-positive bacteria where hydrophilic proteins represent the vast majority of those characterized.

Keywords: Mass spectrometry / Peptide mass mapping / *Streptococcus mutans* / Two-dimensional gel electrophoresis
PRO 0391

1 Introduction

Dental diseases, such as dental caries, are pandemic and result in a significant economic burden in terms of treatment costs, especially in the Western world. Dental caries results from poor oral hygiene coupled with the consumption of a diet rich in fermentable carbohydrates. While dental caries is multifactorial in nature, the disease is associated with the overgrowth of acidogenic and aciduric bacteria, such as *Streptococcus mutans*, a species now well recognized as a major pathogen associated with the disease [1, 2].

Several 2-DE protein analyses of streptococci have been reported [3]. Those involving *S. mutans* have concentrated on physiological aspects associated with its virulence and survival in the oral cavity. In a study of the stress response of *S. mutans*, ¹⁴C-labelled proteins from cells grown in batch culture that had been subjected to pH,

temperature, salt, oxidative and starvation stresses, were displayed [4]. By examining two-fold changes in protein expression between stressed and control cells, *S. mutans* was shown to respond to adverse environmental conditions by a complex and diverse alteration in protein synthesis. Unfortunately, no proteins were identified in this study [4]. Changes in ¹⁴C-labelled cellular proteins following a transition of *S. mutans* from the planktonic to biofilm state in a biofilm chemostat have also been reported. A total of 694 protein spots were analyzed for enhanced or diminished protein expression. Mass spectral analysis resulted in the identification of 41 proteins, some existing as multiple protein spots [5]. In a third study, changes in protein expression associated with the growth of *S. mutans* at low pH were analyzed following comparison of 2-DE proteome maps of *S. mutans* grown in batch culture at two different pH. Thirty individual cellular proteins differing in expression by more than 1.5-fold were identified, including proteins involved in energy metabolism, cell division, translation and transport [6]. What is clear from these studies, is that none has resulted in a comprehensive map of the proteins expressed by *S. mutans* [5, 6]. The recent publishing of contigs of the *S. mutans* genome on a public database (<http://www.genome.ou.edu/smutans.html>) has, however, made such an analysis possible and allowed high resolution prote-

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Abbreviation: PMM, peptide mass mapping

omic maps of *S. mutans* cellular and extracellular proteins to be obtained. By identifying proteins from cells grown in continuous culture under conditions that mimic those in the dental plaque of a healthy individual [7, 8], a baseline for protein expression has been established. This will allow for a far more comprehensive comparative proteomic appraisal of changes in protein expression by *S. mutans* in the near future, along the lines initiated in the studies described above [4–6].

2 Materials and methods

2.1 Chemicals and enzymes

Amino acids, vitamins, adenine, guanine, uracil, mutanolysin, 2-mercaptoethanol, TCA, formic acid, acetone, bacterial protease inhibitor cocktail, chloramphenicol, exonuclease III, thiourea, caprylyl sulfobetaine (*N*-decyl-*N*, *N*-dimethyl-3-ammonia-1-propane-sulfonate; SB 3-10) and α -cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich (Steinheim, Germany). PBS (Dulbecco 'A' tablets), pH 7.3 was obtained from Oxoid (Hampshire, England) and tributylphosphine (97%) was sourced from Fluka (Buchs, Switzerland). IPG strips were obtained from either Amersham Biosciences (Uppsala, Sweden) or Bio-Rad (Hercules, CA, USA). Urea, CHAPS, carrier ampholytes, Tris, bromophenol blue, CBB G-250 were also obtained from Bio-Rad and Sypro Ruby fluorescent dye from Molecular Probes (Eugene, OR, USA). Acetonitrile and NH_4HCO_3 were acquired from Mallinckrodt (Chesterfield, MO, USA), modified porcine sequencing grade trypsin from Promega (Madison, WI, USA), Poros R2 resin from PerSeptive Biosystems (Framingham, MA, USA) and horse blood from Amyl Media (Kings Langley, NSW, Australia). All other reagents were of analytical reagent grade or the highest purity available.

2.2 Bacterial strain, media and growth conditions

S. mutans LT11 [9] was continuously cultured in triplicate at pH 7.0 ± 0.1 and a dilution rate (*D*) of $0.100 \pm 0.001 \text{ h}^{-1}$ as previously described [7] using defined medium with mucin (DMM) medium devoid of mucin but modified to include adenine, guanine and uracil at 20 $\mu\text{g}/\text{mL}$ and both KH_2PO_4 and K_2HPO_4 at 15 mM [10].

2.3 Preparation of cellular proteins

When steady state had been achieved, the contents of the culture vessel were harvested by centrifugation ($10\,000 \times g$, 15 min, 4°C) in the presence of 50 $\mu\text{g}/\text{mL}$

chloramphenicol and 2.5 $\mu\text{L}/\text{mL}$ bacterial protease inhibitor cocktail, washed twice in cold PBS (pH 7.8) containing the same inhibitors and the cell pellet lyophilized. Lyophilized cells (10 mg) were resuspended in 1 mL of 10 mM K_2HPO_4 containing 10 mM MgCl_2 and 1 mM CaCl_2 and incubated at 60°C with 500 U/mL mutanolysin for 40 min. The whole cell lysate was exhaustively dialyzed against 18 M Ω H_2O containing 0.05% w/v NaN_3 , lyophilized, and the proteins extracted and precipitated using a modified TCA-acetone procedure [11]. The precipitate was resuspended in 1 mL of 2-DE solubilizing solution consisting of 5 M urea, 2 M thiourea, 2 mM tributylphosphine, 2% w/v CHAPS, 2% w/v SB 3-10, 0.2% v/v carrier ampholytes (pH 3–10), 40 mM Tris and 0.002% w/v bromophenol blue. The sample was sonicated (Branson, Danbury, USA, output control of 2, $10 \times 10 \text{ s}$, 20–22°C with cooling on ice between each burst) before being incubated at room temperature (20–22°C) for 20 min with 150 U of exonuclease III to degrade any DNA. The solubilized proteins were separated from non-solubilized cellular components by centrifugation ($12\,000 \times g$, 8 min, 15°C) and either used immediately or stored at –80°C until required.

2.4 Preparation of extracellular proteins

The extracellular culture fluid was filtered (MF75 filter units, Nalge Nunc, Rochester, NY, USA) and the proteins recovered using a modification of the method of Chu *et al.* [12]. In essence, culture fluid proteins were precipitated overnight at –20°C with 10% w/v TCA before being centrifuged and washed in ice-cold acetone ($17\,000 \times g$, 30 min, 4°C). After air-drying, the proteins in the pellet were solubilized as described in Section 2.3.

2.5 2-DE

Cellular proteins extracted from both 0.5 and 0.75 mg dry weight of cells from each of the three chemostats were dissolved in 450 μL of 2-DE solubilizing solution and either loaded by in-gel rehydration onto pH 4–7 IPG strips or cup-loaded onto narrow range (pH 4.0–5.0, 4.5–5.5 and 5.5–6.7) or basic pH 6–11 IPG strips preswollen with 350 μL of 2-DE solubilizing solution. For the analysis of the culture fluid, a 13-fold concentration or approximately 100 μg of precipitated protein from each of the three chemostats was dissolved in 460 μL of 2-DE solubilizing solution and the solution used to rehydrate pH 4–7 IPG strips. The cellular and culture fluid proteins were focused in triplicate on broad range pH 4–7, and in duplicate on narrow range IPG strips (as appropriate) using a Multiphor II (Amersham Biosciences) at 20°C. Proteins were focused for a total of 71.1 kVh before separation in the second dimension using a Protean II xi system (Bio-Rad)

as previously described [13]. The gels were then fixed and stained with Sypro Ruby for imaging with a Molecular Imager Fx (Bio-Rad) before being 'double stained' with CBB G-250 [14].

2.6 Protein identification

Protein spots were excised from the gel and individually digested with trypsin as previously described [14]. A 1 µL aliquot of each digest was spotted onto a MALDI target plate (Micromass, Manchester, UK), and 0.5 µL of matrix solution (10 mg α-cyano-4-hydroxycinnamic acid per ml 70% v/v acetonitrile) layered on top and allowed to dry. In some instances, microscale concentration and desalting was performed using C₁₈ Zip-Tips™ (Millipore, Bedford, MA, USA) or micropurification columns employing narrow diameter GelLoader™ tips (Eppendorf, Hamburg, Germany) packed with Poros R2 chromatography resin [15, 16]. Zip-Tips™ and R2 columns were activated with 20 µL of 100% v/v acetonitrile and equilibrated with 20 µL 5% v/v formic acid. Peptides were eluted from the columns with 1 µL matrix solution onto a MALDI target plate.

Peptide mass mapping (PMM) analyses of proteins digested with trypsin were performed on a Micromass TOF-Spec 2E or a PerSeptive Biosystems Voyager DE-STR mass spectrometer with trypsin autolysis peptide masses of 842.5 and 2211.1 Da being used as internal standards. All mass spectra were obtained in reflectron-delayed extraction mode. Peak list generation made use of either Data Explorer™ (PerSeptive Biosystems) or ProteinLynx (Micromass) software. All database searching with PMM made use of the ProteinLynx software package containing the unpublished five contigs of the *S. mutans* UA159 genome (<http://www.genome.ou.edu/smutans.html>) downloaded on October 6, 2001 that were translated in all six reading frames using the Flip6Frames program (17; <http://megasun.bch.umontreal.ca/ogmp/ogmpid.html>) accessed through the Australian Genomic Information Service (<http://www.angis.org.au/WebANGIS>). Parameters for protein identification included a mass tolerance of 150 ppm and a maximum of one missed cleavage *per* peptide with the methionine sulfoxide and cysteine acrylamide modification options on. Matches were defined on the basis of the number of matching peptide masses and the total percentage sequence covered by the peptides. As a general rule, a minimum total sequence coverage of 25% was taken to match a given translated ORF with confidence, with the proviso that cleavage products and low and high mass proteins did not always conform to this rule. All translated ORFs that matched PMM data were used to query the SWISS-PROT and TrEMBL databases using the FASTA program (<http://www2.ebi.ac.uk/fasta3/>).

Tandem ESI-MS/MS was performed using a Q-tof hybrid quadrupole/orthogonal-acceleration TOF mass spectrometer (Micromass) as previously described [14] with the amino acid sequences being derived from the mass differences between the *y*- or *b*-ion 'ladder' series. Peptide sequences were then used to query the *S. mutans* UA159 translated contigs as well as the incomplete genome sequences of the related species *Streptococcus pneumoniae*, *Streptococcus gordonii*, *Streptococcus equis* and *Streptococcus pyogenes* using the blastp program (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table.cgi). Amino acid sequences were also used to search the completed genomes in the SWISS-PROT and TrEMBL databases using FASTA.

3 Results and discussion

3.1 Analysis of the *S. mutans* proteome by 2-DE

The use of standardized continuous culture conditions and the availability of contigs of the *S. mutans* UA159 genome allowed a cellular and extracellular proteomic map of *S. mutans* LT11 to be constructed. The conditions chosen were those of neutral pH and a bacterial doubling time of approximately 7 h that were consistent with the conditions associated with a resting dental plaque in between meal times [7, 8]. Glucose was chosen as the source of carbon as this carbohydrate catabolite represses the expression of other genes [18], thus allowing for future *in vitro* modelling of pathways expressed when the source of carbon is altered, such as when sucrose is ingested during food intake.

A number of different methods for extracting proteins from chemostat grown *S. mutans* were trialed (data not shown), as oral Gram-positive streptococci are difficult to lyse and require a specific muramidase to disrupt the integrity of the peptidoglycan matrix of the cell wall. The final protocol that was adopted included digestion of the wall sacculus with the muramidase, mutanolysin [19], followed by TCA-acetone extraction prior to protein solubilization.

Image analysis, using the software package z3 (Compu-gen, Tel Aviv, Israel), of triplicate 2-DE gels displaying cellular proteins derived from separate chemostat cultures showed little pattern deviation that could be attributed to either sample or run-to-run variation (data not shown). The total number of spots visible on the 2-DE gels was approximately 1050 and 200 over the pH ranges 4.0–7.0 and 6.0–11.0 respectively (Fig. 1). Protein resolution was further enhanced using narrow pH range (pH 4.0–5.0, 4.5–5.5 and 5.5–6.7) IPG strips to resolve clusters of proteins with near identical p/s in the acidic pH range of 4.5–6.0.

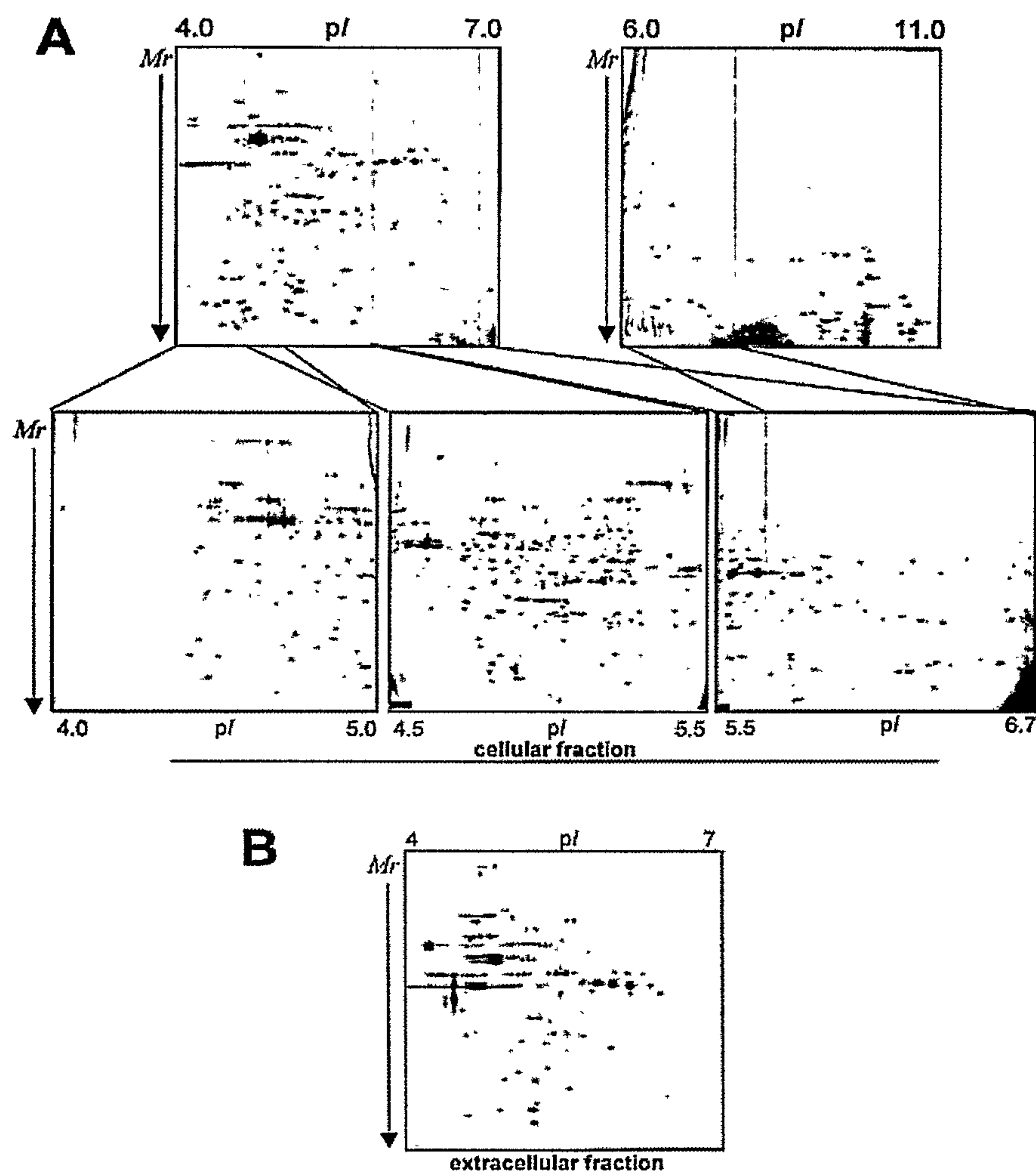


Figure 1. 2-DE proteomic maps of *S. mutans* LT11 grown in a glucose limited chemostat at pH 7.0. (A) Soluble cellular proteins were focused in the first dimension using mid-range (pH 4.0–7.0 and 6.0–11.0) and narrow range (pH 4.0–5.0, 4.5–5.5 and 5.5–6.7) IPG strips. The corresponding 1.0 pH unit regions of the first dimension narrow range IPG strips are shown in relation to their broad pH range 4.0–7.0 and 6.0–11.0 counterparts. (B) TCA precipitated proteins from the culture fluid were focused over the pH range 4.0–7.0 prior to separation in the second dimension.

The number of spots detected by z3 software on pH range 4.0–5.0, 4.5–5.5 and 5.5–6.7 2-DE gels was of the order of 800, 1300 and 650 respectively, or approximately twice the number of spots detected in the corresponding regions of the pH 4–7 2-DE gels after allowing for overlapping regions (Fig. 1). A similar observation was made following 2-DE analysis of *Staphylococcus aureus* where the use of narrow range IPG strips increased the number of protein spots that were resolved by 45% [20]. Soluble proteins present in the culture fluid were also focused over the pH range 4.0–7.0. Image analysis revealed highly reproducible 2-DE gels displaying approximately 900 spots (Fig. 1).

3.2 Identification of *S. mutans* proteins

A total of 502 protein spots that could be visualized by eye following double staining in Sypro Ruby and CBB G-250 were obtained from the cellular and extracellular

2-DE gels over the pH ranges 4.0–11.0 and 4.0–7.0 respectively (Figs. 2 and 3). All 502 proteins were digested with trypsin and analyzed by MALDI-TOF MS. Of these proteins, 416 (313 cellular and 103 in the culture fluid; 83%) provided PMM spectra of sufficient quality to perform a database search of the translated ORFs of the *S. mutans* contigs. The protein spots that could not be characterized were either of insufficient concentration for analysis following purification, or the number of peptides generated by trypsin and characterized by MALDI-TOF MS were insufficient to positively identify the protein. The majority of these proteins were of low abundance and low M_r .

The 416 proteins that were identified by PMM matched 197 translated ORFs within the *S. mutans* contigs. These translated ORFs were subsequently used to search for homologues in the SWISS-PROT and TrEMBL databases, and where matches were obtained, a putative function

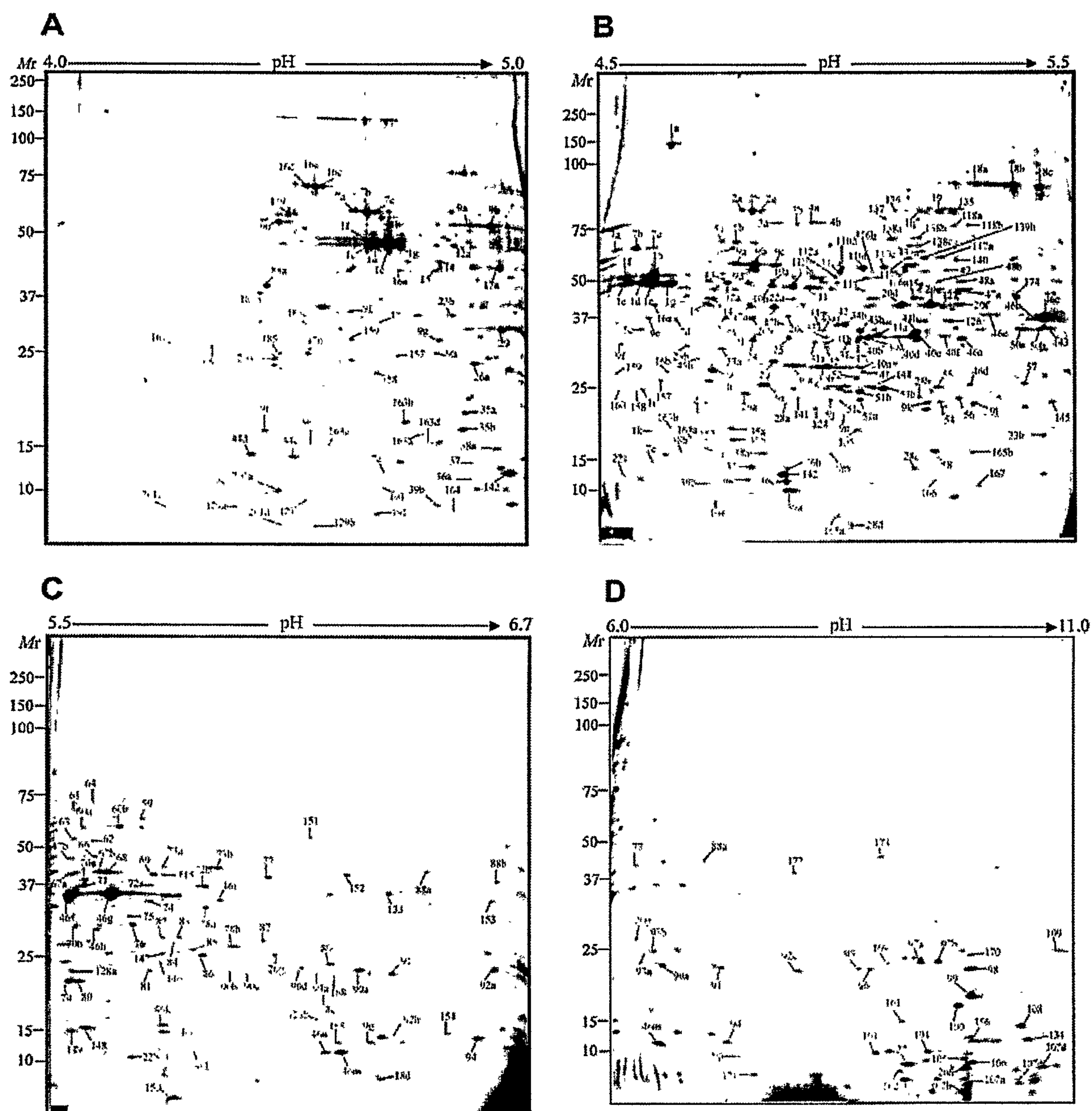


Figure 2. Solubilized proteins from cells of *S. mutans* LT11 that were identified by MALDI-TOF MS or ESI-MS/MS following separation by 2-DE. Proteins focused over the pH range 4.0–5.0 (A), 4.5–5.5 (B), 5.5–6.7 (C) and 6.0–11.0 (D) and stained with Sypro Ruby. The numbers refer to the proteins that have been identified (see Table 1).

was assigned and the proteins categorized according to their function (Table 1). Thirteen proteins that could not be identified by PMM, were subjected to ESI-MS/MS. Only five provided peptide amino acid sequences. These sequences matched four different proteins in the SWISS-PROT, TrEMBL and unfinished microbial genome databases (Table 2).

Although a total of 200 different proteins were identified, only 34 matched proteins that had been previously characterized from *S. mutans*, meaning that 166 novel proteins were identified in this study. Not unexpectedly, the closest homologues of these proteins were usually those from other streptococci, particularly *S. pneumoniae* and *S. pyogenes* (Table 1). A comparison of the cellular and

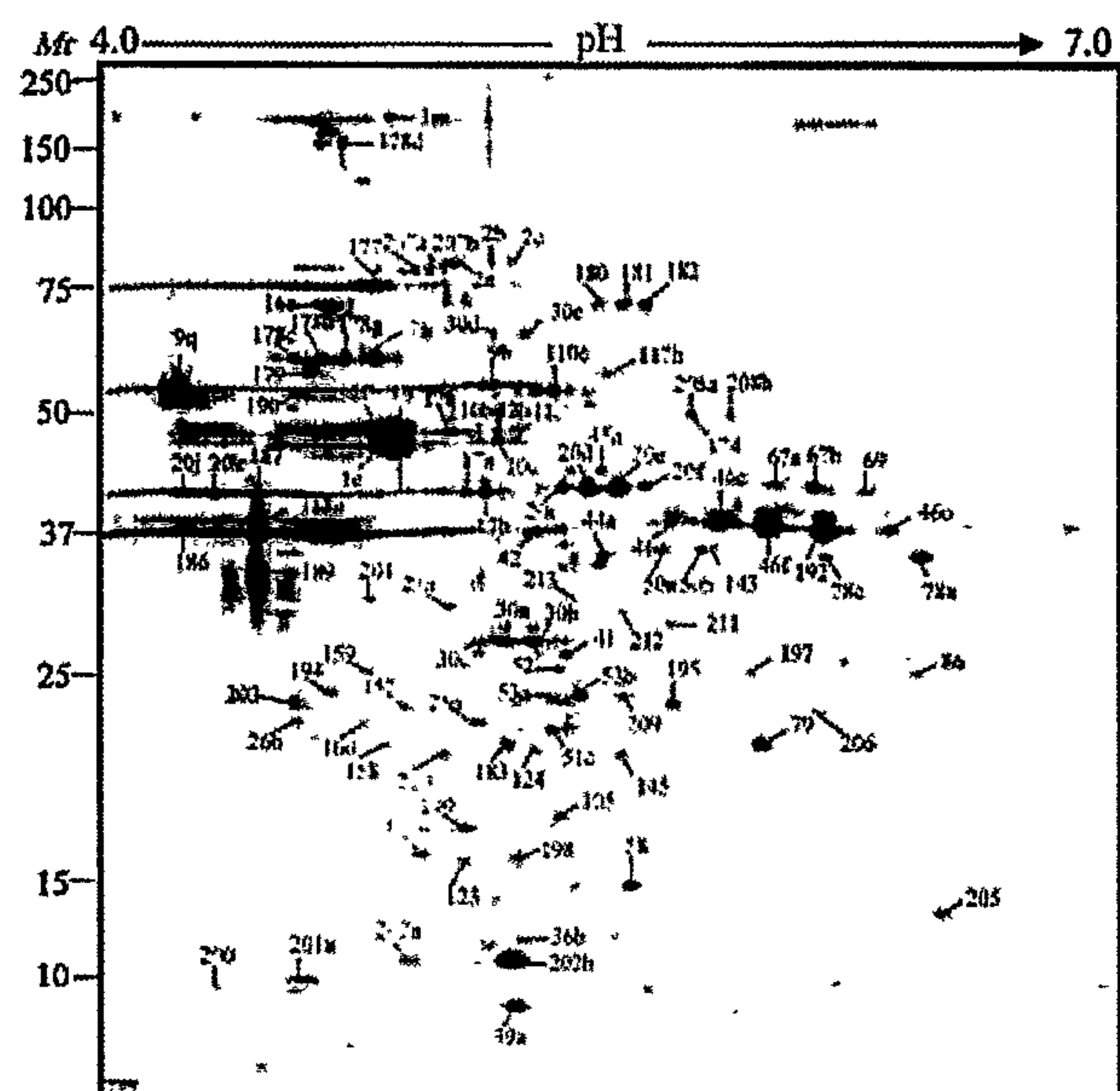


Figure 3. TCA precipitated proteins from the culture fluid of chemostat grown *S. mutans* LT11 that were identified by MALDI-TOF MS or ESI-MS/MS following separation by 2-DE over the pH range 4.0–7.0. The numbers refer to the proteins that have been identified (see Table 1).

culture fluid proteins showed that 15% of those identified (including their modified forms) were found in both compartments. Of the 172 different proteins identified from the cellular fraction, 26 (15%) were functionally undefined. The majority of the identified proteins were associated with house keeping functions such as energy metabolism, amino acid biosynthesis, translation and transport, and binding (Table 1).

Sixty-four proteins detected in both the cellular and extracellular compartments showed isoelectric heterogeneity (charged isoforms) and/or varied in mass. Of the 35 proteins that were detected as pairs of protein spots, 26 were charged isoforms of one another. Isoelectric heterogeneity is considered to be due to post-translational modification of proteins, such as phosphorylation, glycosylation or acylation, or often as the result of an artefact including urea carbamylation or deamidation [22–26]. The nature of any modification of *S. mutans* proteins observed in the current analysis remains to be determined. The four most prominently expressed proteins, three glycolytic enzymes and the translation elongation factor Tu, however, were detected as more than ten protein spots many of which were lower M_r forms (Table 1). Of these, the glycolytic enzyme, enolase, was of particular interest as it formed a cluster of six double banded variants within the pI range 4.5–4.6 (proteins 1b, c, d, e, f and g; Figs. 2B and 3), and also existed in two higher mass forms, one approximately

three times (protein 1a; Fig. 2B) and the other approximately twice the predicted molecular mass and with a more basic pI of 5.2 (protein 1h; Fig. 2B). Similar multi-metric mass variants have been observed in other bacteria including *S. aureus* [14] and *Escherichia coli* [21].

Only three *S. mutans* wall-associated proteins, WapA, Gbp and SpaP (proteins 189, 177 and 136 respectively; Figs. 2B and 3) were identified, the former two in the extracellular compartment and the last as an N-terminal portion of its C-terminally anchored form in the cellular fraction. A single integral membrane protein was detected in the culture fluid. This was an internal fragment of a homologue of the branched chain amino acid transporter, LivJ (protein 205; Fig. 3). Although membrane proteins represent around 30% of total proteins of bacteria [27], membrane-embedded or intrinsic cytoplasmic membrane proteins are rarely, if at all, identified in 2-DE proteomic maps even in membrane-enriched fractions. This is due to their poor solubility and inherent hydrophobicity. This should be contrasted with membrane-enriched fractions from Gram-negative bacteria which allow the detection of lipoproteins and outer membrane (OM) proteins by 2-DE [13, 28–35]. Whereas the transmembrane domains of OM proteins possess β -pleated sheets formed from alternating polar and nonpolar amino acids, integral cytoplasmic membrane proteins generally possess hydrophobic α -helical transmembrane domains consisting of nonpolar amino acids. Intrinsic membrane proteins therefore, maintain an overall hydrophobicity that is hydrophobic even when denatured [27]. Unfortunately, solubilization of denatured intrinsic cytoplasmic membrane proteins requires the use of surfactants such as SDS which are incompatible with IPG strips. Clearly, different methods of solubilization and/or new methods for selecting or enriching for peptides and proteins associated with cytoplasmic membranes are required for any comprehensive 2-DE display, irrespective of the cell type [27].

PMM analysis identified 103 proteins in the culture fluid (Fig. 3), 60 of which were also detected in the cytosolic fraction. Of the remaining 43 proteins, 27 are generally considered to be of cellular origin even though they were not detected in the cellular fraction and four were 'hypothetical' proteins with no known cellular location (Table 1, protein spots 198, 204, 212 and 213). Four additional protein spots present in the extracellular compartment, but clearly of cellular origin, were identified by peptide ESI-MS/MS (Table 2). Consequently, 91 of the 107 proteins identified in the culture fluid would classically be considered to be associated with the cytoplasm or the inner surface of the cytoplasmic membrane, suggesting that they may be the byproducts of cell lysis. However, it has

recently become apparent that a number of streptococcal proteins previously thought to be confined to the cytosol are also associated with the cell-surface or secreted into the external milieu [36, 37]. For example, in *Streptococcus agalactiae*, many of the major surface-bound proteins have glycolytic or chaperonin functions. They include ornithine carbamoyltransferase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, purine nucleoside phosphorylase, enolase, and glucose-6-phosphate isomerase [38]. Glyceraldehyde-3-phosphate dehydrogenase is of particular interest as it has been found as a surface-associated protein in every streptococcal species examined so far [36] and is implicated as a virulence determinant aiding *S. pyogenes* invasion of tissues [37, 39]. In *S. gordonii*, the glyceraldehyde-3-phosphate dehydrogenase, primarily associated with the cell surface at pH 6.5, becomes the major extracellular protein at pH 7.5 [37]. This may also be the case for the form of the *S. mutans* enzyme observed in the culture fluid in this study. Other than *S. agalactiae*, surface-bound enolases have been found on a number of streptococci including *S. pyogenes* where the enzyme displays strong plasmin(ogen) binding [39, 40]. How these proteins become associated with the cell-surface without one of the known Gram-positive cell-surface anchoring mechanisms [41–43], has yet to be determined, although it has been suggested that *N*-terminal attachment and charge/hydrophobic interactions may be responsible [44].

4 Concluding remarks

This study has established the first comprehensive proteomic reference map of the proteins of *S. mutans*. As the map is based on a standardized continuous culture condition, it allows for future comparative analyses with other *S. mutans* proteomes derived from modelling environmental conditions relevant to the pathogenesis of this organism. By identifying the proteins expressed from 200 ORFs, or approximately 11% of those present in the genome of *S. mutans* UA159, a significant contribution has been made towards complementing the genomic sequencing initiative of this oral bacterium.

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Table 1. Proteins identified from *S. mutans* LT11 categorized into putative functional groups

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
I AMINO ACID BIOSYNTHESIS							
1 Aromatic amino acid family							
87	<i>S. pneumoniae</i>	Shikimate 5-dehydrogenase (<i>aroE</i>)	9		41		2C
126	<i>S. pneumoniae</i>	Phospho-2-dehydro-3-deoxyheptonate aldolase (<i>sp1701</i>)	7		30		2B
151	<i>S. pneumoniae</i>	Prephenate dehydrogenase (<i>tyrA</i>)	4		15		2C
152	<i>S. pneumoniae</i>	Chorismate synthase (<i>sp1374</i>)	8		31		2C
2 Aspartate family							
41	<i>S. pneumoniae</i>	Dihydrodipicolinate reductase (<i>sp1555</i>)	8	9	43	44	2B, 3
49	<i>S. pneumoniae</i>	S-adenosylmethionine synthetase (<i>sp0762</i>)	10		28		2B
127	<i>S. mutans</i>	Aspartate-semialdehyde dehydrogenase (<i>asd</i>)	7		34		2B
147	<i>S. pyogenes</i>	MTA/SAH nucleosidase (<i>pfs</i>)	5		36		2C
208-a	<i>S. pneumoniae</i>	Threonine synthase (<i>thrC</i>)		8		20	3
208-b	<i>S. pneumoniae</i>	Threonine synthase (<i>thrC</i>)		8		19	3
3 Branched chain amino acid family							
21	<i>B. halodurans</i>	Acetoin dehydrogenase e1 component (tpp-dependent beta submit) (<i>acoB</i>)	9		35		2B
34-a	<i>S. thermophilus</i>	Ketol-acid reductoisomerase (<i>ilvC</i>)	7		30		2B
34-b	<i>S. thermophilus</i>	Ketol-acid reductoisomerase (<i>ilvC</i>)	8		34		2B
42	<i>S. pneumoniae</i>	Branched-chain amino acid amino-transferase (<i>sp0856</i>)	8	11	30	40	2B, 3
4 Glutamate family							
174	<i>C. perfringens</i>	NADP-Specific glutamate dehydrogenase (<i>gdhA</i>)	10	9	31	20	2B, 3
5 Serine family							
45-a	<i>L. lactis</i>	Phosphoserine aminotransferase (<i>serC</i>)	8	11	34	32	2B, 3
45-b**	<i>L. lactis</i>	Phosphoserine aminotransferase (<i>serC</i>)	3		14		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
63	<i>S. mutans</i>	Phosphoglycerate dehydrogenase (<i>serA</i>)	5		11		2C
70-a	<i>L. lactis</i>	D-3-Phosphoglycerate dehydrogenase (<i>serA</i>)	8		28		2C
70-b	<i>L. lactis</i>	D-3-Phosphoglycerate dehydrogenase (<i>serA</i>)	8		29		2C
78-a	<i>S. suis</i>	O-acetylserine lyase (<i>cysM</i>)	8	15	46	62	2C, 3
78-b	<i>S. suis</i>	O-acetylserine lyase (<i>cysM</i>)	10		55		2C
78-c	<i>S. suis</i>	O-acetylserine lyase (<i>cysM</i>)		11		47	3
II CELL ENVELOPE							
1 Murein sacculus and peptidoglycan							
66	<i>S. pyogenes</i>	Udp-N-acetyl muramate-alanine ligase (<i>murC</i>)	12		45		2C
140	<i>S. algalactiae</i>	D-Alanine-D-alanyl carrier protein ligase (<i>dltA</i>)	11		28		2B
150***	<i>S. pneumoniae</i>	Peptidoglycan N-acetyl-glucosamine deacetylase A (<i>sp1479</i>)	5		20		2C
195	<i>S. pyogenes</i>	Putative N-acetylglucosamine-6-phosphate isomerase (<i>nagB</i>)		9		50	3
2 Surface antigens							
136*	<i>S. mutans</i>	Cell surface antigen I/II precursor (<i>spaP</i>)	7		6		2B
177	<i>S. mutans</i>	Glucan-binding protein precursor (<i>gbp</i>)		11		31	3
189	<i>S. mutans</i>	Chain 1: Wall-associated protein (<i>wapA</i>)		5		23	3
III CELLULAR PROCESSES							
1 Cell division							
111	<i>S. pyogenes</i>	Cell division protein (<i>ftsA</i>)	13		43		2B
162**	<i>S. pyogenes</i>	Chromosome segregation protein (<i>smc</i>)	4		5		2B
170	<i>S. pyogenes</i>	Cell-division ATP-binding protein (<i>ftsE</i>)	9		42		2D
188-a	<i>S. pyogenes</i>	Cell-division initiation protein (septum placement) (<i>spy1514</i>)	10	11	48	41	2A, 3
188-b	<i>S. pyogenes</i>	Cell-division initiation protein (septum placement) (<i>spy1514</i>)	6		38		2A
188-c	<i>S. pyogenes</i>	Cell-division initiation protein (septum placement) (<i>spy1514</i>)	5		35		2A
190	<i>S. pyogenes</i>	Cell division protein (<i>ftsZ</i>)	13	10	46	30	2A, 3
2 Chaperones							
7-a	<i>S. pyogenes</i>	60 kDa chaperonin (<i>groEL</i>)	18		42		2A, 2B
7-b	<i>S. pyogenes</i>	60 kDa chaperonin (<i>groEL</i>)	25	16	55	36	2A, 2B
7-c	<i>S. pyogenes</i>	60 kDa chaperonin (<i>groEL</i>)	15		38		2A, 2B
7-d*	<i>S. pyogenes</i>	60 kDa chaperonin (<i>groEL</i>)	8		21		2B
7-e***	<i>S. pyogenes</i>	60 kDa chaperonin (<i>groEL</i>)	5		13		2A, 2B
16-a*	<i>S. algalactiae</i>	Chaperone protein DnaK (heat shock protein 70) (<i>dnaK</i>)	7		15		2A, 2B
16-b*	<i>S. algalactiae</i>	Chaperone protein DnaK (heat shock protein 70) (<i>dnaK</i>)	9		21		2B
16-c	<i>S. algalactiae</i>	Chaperone protein DnaK (heat shock protein 70) (<i>dnaK</i>)	22	16	52	30	2A, 3

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
16-d	<i>S. algalactiae</i>	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	14		28		2A
16-e	<i>S. algalactiae</i>	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	9		25		2A
16-f**	<i>S. algalactiae</i>	Chaperone protein DNAK (heat shock protein 70) (<i>dnaK</i>)	4		13		2A
36-a	<i>S. pneumoniae</i>	10 kDa chaperonin (<i>sp1907</i>)	4		38		2A, 2B
36-b	<i>S. pneumoniae</i>	10 kDa chaperonin (<i>sp1907</i>)	4	6	38	47	2B, 3
36-c	<i>S. pneumoniae</i>	10 kDa chaperonin (<i>sp1907</i>)	6		75		2B
179	<i>S. pyogenes</i>	Trigger factor (<i>tig</i>)	15	11	45	42	2A, 3
3 Detoxification							
124	<i>S. mutans</i>	Superoxide dismutase [Mn/Fe] (<i>sodA</i>)	6	5	40	30	2B, 3
4 Protein and peptide secretion							
142**	<i>B. halodurans</i>	Signal peptidase-like protein (<i>bh0045</i>)	4		16		2A, 2B
5 Transformation							
154	<i>S. pyogenes</i>	Late competence protein required for DNA binding (<i>comEB</i>)	4		32		2C
IV CENTRAL INTERMEDIARY METABOLISM							
1 General							
68	<i>S. pneumoniae</i>	Required for glycogen biosynthesis (<i>glgD</i>)	12		40		2C
2 Amino acids							
138-a	<i>S. pyogenes</i>	L-glutamine-D-fructose-6-phosphate amidotransferase (<i>glmS</i>)	10		22		2B
138-b	<i>S. pyogenes</i>	L-glutamine-D-fructose-6-phosphate antidotransferase (<i>glmS</i>)	15		34		2B
138-c	<i>S. pyogenes</i>	L-glutamine-D-fructose-6- phosphate amidotransferase (<i>glmS</i>)	5		13		2B
V ENERGY METABOLISM							
1 Aerobic							
56	<i>S. pyogenes</i>	NADH dehydrogenase (<i>spy1069</i>)	6		41		2B
96	<i>S. pyogenes</i>	NAD(P)H-flavin oxidoreductase (<i>spy0512</i>)	6		25		2D
74	<i>S. mutans</i>	GpdA (<i>gpdA</i>)	7		33		2C
2 ATP-proton motive force interconversions							
155	<i>S. mutans</i>	ATP synthase epsilon chain (<i>atpC</i>)	4		44		2C
3 Fatty acid and phospholipid metabolism							
67-a	<i>S. pneumoniae</i>	Beta-ketoacyl-ACP synthase II (<i>fabF</i>)	6	7	28	27	2C, 3
67-b	<i>S. pneumoniae</i>	Beta-ketoacyl-ACP synthase II (<i>fabF</i>)	13	7	54	28	2C, 3
210**	<i>S. pyogenes</i>	Acyltransferase (<i>spy0041</i>)		10		19	3
4 Fermentation							
18-a	<i>L. lactis</i>	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	7		12		2B
18-b	<i>L. lactis</i>	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	5		10		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
18-c	<i>L. lactis</i>	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	9		16		2B
18-d***	<i>L. lactis</i>	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	4		6		2C
18-e***	<i>L. lactis</i>	Alcohol-acetaldehyde dehydrogenase (<i>adhE</i>)	4		8		2C
25	<i>S. pyogenes</i>	Phosphotransacetylase (<i>pta</i>)	8		31		2B
69	<i>S. pneumoniae</i>	Acetate kinase (<i>ackA</i>)	12	10	41	30	2C, 3
85	<i>L. lactis</i>	Acetoin reductase (<i>butA</i>)	8		52		2C
135	<i>S. mutans</i>	Formate acetyltransferase (<i>pfl</i>)	13		21		2B
5 Glycogen synthesis							
14	<i>S. pneumoniae</i>	Glucose-1-phosphate adenylyltransferase (<i>sp1122</i>)	8		29		2B, 2A
6 Glycolysis							
1-a	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	15		48		2B
1-b	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	18	10	52	30	2B, 3
1-c	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	18	9	56	29	2B, 3
1-d	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	14		50		2B
1-e	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	12	9	45	27	2B, 3
1-f	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	13	14	47	47	2B, 3
1-g	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	12		46		2B
1-h	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	11		41		2B
1-i**	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	5		15		2B
1-j**	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	5		18		2B
1-k*	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	4		17		2B
1-l*	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	3		10		2C
1-m	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)		13		35	3
1-n*	<i>S. pneumoniae</i>	Enolase (<i>eno</i>)	3		15		2A
5-a	<i>S. thermophilus</i>	Phosphoglucosmutase (<i>pgmA</i>)	15		40		2B
5-b	<i>S. thermophilus</i>	Phosphoglucosmutase (<i>pgmA</i>)	25		63		2B
5-c*	<i>S. thermophilus</i>	Phosphoglucosmutase (<i>pgmA</i>)	7		18		2B
12-a	<i>S. mutans</i>	Glucose-6-phosphate isomerase (<i>gpi</i>)	17	17	50	45	2B, 3
12-b	<i>S. mutans</i>	Glucose-6-phosphate isomerase (<i>gpi</i>)		6		13	3
20-a	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	10		32		2B
20-b	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	14		46		2B
20-c	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	8		25		2B
20-d	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	18	6	53	19	2B, 3
20-e	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	16	6	51	19	2B, 3
20-f	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	8	6	25	23	2B, 3
20-g	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	8		25		2C, 2D
20-h	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)	5		13		2D
20-i	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)		16		44	3
20-j	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)		13		33	3
20-k	<i>S. pneumoniae</i>	Phosphoglycerate kinase (<i>pgk</i>)		21		66	3
30-a	<i>S. bovis</i>	Class-II aldolase (<i>fba</i>)	11	6	48	41	2B, 3
30-b	<i>S. bovis</i>	Class-II aldolase (<i>fba</i>)	10	8	38	30	2B, 3

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
30-c	<i>S. bovis</i>	Class-II aldolase (<i>fba</i>)		7		41	3
30-d	<i>S. bovis</i>	Class-II aldolase (<i>fba</i>)		7		33	3
30-e	<i>S. bovis</i>	Class-II aldolase (<i>fba</i>)		9		43	3
43-a	<i>S. mutans</i>	L-lactate dehydrogenase (<i>ldh</i>)	6		26		2B
43-b	<i>S. mutans</i>	L-lactate dehydrogenase (<i>ldh</i>)	9		31		2B
46-a	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	11		39		2B
46-b	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	9		23		2B
46-c	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	11	10	29	27	2B, 3
46-d**	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	5		20		2B
46-e	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	10	13	36	37	2B, 3
46-f	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	12	10	40	27	2C, 3
46-g	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	17		43		2C
46-h	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	10		32		2C
46-l	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	6		16		2C
46-j*	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	5		14		2C
46-k*	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	4		10		2C
46-l*	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	6		19		2C
46-m**	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	7		22		2C, 2D
46-n*	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)	4		12		2C
46-o*	<i>S. pneumoniae</i>	Glyceraldehyde 3-phosphate dehydrogenase (<i>sp2012</i>)		7		22	3
50-a	<i>S. thermophilus</i>	6-phosphofructokinase (<i>pfkA</i>)	7	7	23	18	2B, 3
50-b	<i>S. thermophilus</i>	6-phosphofructokinase (<i>pfkA</i>)	9	7	34	18	2B, 3
51-a	<i>L. lactis</i>	Triosephosphate isomerase (<i>tpiA</i>)	8		44		2B
51-b	<i>L. lactis</i>	Triosephosphate isomerase (<i>tpiA</i>)	5		33		2B
51-c	<i>L. lactis</i>	Triosephosphate isomerase (<i>tpiA</i>)	7	7	44	44	2B, 3
53-a	<i>S. pneumoniae</i>	Phosphoglyceromutase (<i>gpmA</i>)	4	8	21	32	2B, 3
53-b	<i>S. pneumoniae</i>	Phosphoglyceromutase (<i>gpmA</i>)	11	12	42	56	2B, 3
110-a	<i>S. pyogenes</i>	Pyruvate kinase (<i>pyk</i>)	14		40		2B
110-b	<i>S. pyogenes</i>	Pyruvate kinase (<i>pyk</i>)	12		28		2B
110-c	<i>S. pyogenes</i>	Pyruvate kinase (<i>pyk</i>)	12	11	33	22	2B, 3
110-d	<i>S. pyogenes</i>	Pyruvate kinase (<i>pyk</i>)	18		48		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover- age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
7 Pentose phosphate pathway							
4-a	<i>S. pneumoniae</i>	Transketolase (<i>tkt</i>)	8		17		2B
4-b	<i>S. pneumoniae</i>	Transketolase (<i>tkt</i>)	13		26		2B
157	<i>S. pyogenes</i>	Ribose 5-phosphate isomerase (<i>rpiA</i>)	11	8	51	43	2B, 3
8 Pyruvate dehydrogenase							
62	<i>S. pyogenes</i>	Dihydrolipoamide dehydrogenase, component E3 (<i>acoL</i>)	6		26		2C
9 Sugars							
24	<i>S. pyogenes</i>	Glucose kinase (<i>glcK</i>)	9		35		2B
61	<i>S. pyogenes</i>	4- α -glucanotransferase (<i>malQ</i>)	5		15		2C
143	<i>S. mutans</i>	UDP-glucose 4-epimerase (<i>galE</i>)	9	8	35	31	2B, 3
211	<i>S. mutans</i>	Fructokinase (<i>scrK</i>)		4		21	3
10 TCA cycle							
11	<i>S. pyogenes</i>	Succinic semialdehyde dehydrogenase (<i>gabD</i>)	13	6	31	14	2B, 3
160*	<i>S. pneumoniae</i>	2,3,4,5-Tetrahydropyridine-2- carboxylate <i>N</i> -succinyltransferase-related protein (<i>gapD</i>)	5	8	33	43	2B, 3
VI FATTY ACID METABOLISM							
76	<i>S. pyogenes</i>	Trans-2-enoyl-acp reductase II (<i>spyM18_1823</i>)	9		46		2C
92-a	<i>S. pyogenes</i>	Beta-ketoacyl-acp reductase (<i>fabG</i>)	7		44		2C
92-b*	<i>S. pyogenes</i>	Beta-ketoacyl-acp reductase (<i>fabG</i>)	9		42		2C
92-c	<i>S. pyogenes</i>	Beta-ketoacyt-acp reductase (<i>fabG</i>)	9		45		2D
101	<i>S. pneumoniae</i>	(3R)-Hydroxymyristoyl-[acyl carrier protein] dehydratase (<i>fabZ</i>)	6		35		2D
VII PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES							
1 Purine ribonucleotide biosynthesis							
60-a	<i>S. pyogenes</i>	Inosine-5'-monophosphate dehydro- genase (<i>guaB</i>)	11		29		2C
60-b	<i>S. pyogenes</i>	Inosine-5'-monophosphate dehydro- genase (<i>guaB</i>)	14		34		2C
2 Pyrimidine ribonucleotide biosynthesis							
57	<i>S. pyogenes</i>	Uridylate kinase (<i>pyrH</i>)	6		37		2B
3 Salvage of nucleotides and nucleosides							
141	<i>S. pyogenes</i>	Guanylate kinase (<i>gmk</i>)	8		36		2B
168	<i>S. salivarius</i>	Uracil phosphoribosyl-transferase (<i>upp</i>)	8		56		2C
206	<i>S. mutans</i>	Deoxyribose-phosphate aldolase (<i>deoC</i>)		4		24	3
209	<i>S. pyogenes</i>	Adenylate kinase (<i>adk</i>)		7		42	3
4 Sugar-nucleotide biosynthesis and interconversions							
29	<i>S. mutans</i>	Glucose-1-phosphate thymidyltransferase (<i>rmlA</i>)	15		52		2A, 2B
32	<i>S. mutans</i>	DTDP-4-keto-L-rhamnose reductase (<i>rmlD</i>)	6		27		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
71	<i>S. mutans</i>	DTDP-Glucose 4,6-dehydratase (<i>rmIB</i>)	9		32		2C
183	<i>S. mutans</i>	DTDP-4-keto-L-rhamnose reductase (<i>rmIC</i>)		8		50	3
VIII REGULATORY FUNCTIONS							
1 General							
55	<i>S. mutans</i>	GcrR protein (<i>gcr</i>)	7		44		2B
28-a	<i>S. pyogenes</i>	Cell-cycle regulation histidine triad protein (<i>hit</i>)	4		31		2B
28-b	<i>S. pyogenes</i>	Cell-cycle regulation histidine triad protein (<i>hit</i>)	4		30		2B
28-c	<i>S. pyogenes</i>	Cell-cycle regulation histidine triad protein (<i>hit</i>)	8		75		2B
28-d*	<i>S. pyogenes</i>	Cell-cycle regulation histidine triad protein (<i>hit</i>)	3		30		2B
58	<i>B. subtilis</i>	ydcP protein (<i>ydcP</i>)	5		50		2B
185	<i>S. pyogenes</i>	Phosphoprotein phosphatase (<i>spyM18_1635</i>)	3		19		2A
2 Lac-I family regulators							
133	<i>S. mutans</i>	Catabolite control protein A (<i>ccpA</i>)	9		40		2C
IX REPLICATION							
1 DNA replication, restriction, modification, recombination and repair							
13	<i>S. pyogenes</i>	DNA polymerase III delta prime subunit (<i>hoIB</i>)	9		27		2B
47-a	<i>S. parasanguis</i>	Recombinase A (<i>recA</i>)	9		35		2B
47-b	<i>S. parasanguis</i>	Recombinase A (<i>recA</i>)	8		31		2C
107-a	<i>S. mutans</i>	DNA-binding protein HU (<i>hup</i>)	5		50		2D
107-b	<i>S. mutans</i>	DNA-binding protein HU (<i>hup</i>)	5		50		2D
107-c	<i>S. mutans</i>	DNA-binding protein HU (<i>hup</i>)	7		67		2D
107-d	<i>S. mutans</i>	DNA-binding protein HU (<i>hup</i>)	4		45		2D
165-a*	<i>S. mutans</i>	Mutator mutt protein (<i>mutX</i>)	3		21		2B
165-b	<i>S. mutans</i>	Mutator mutt protein (<i>mutX</i>)	4		30		2B
181	<i>L. lactis</i>	Replication protein (<i>dnaB</i>)		8		24	3
2 Degradation of DNA							
75	<i>S. mutans</i>	Nuclease (<i>smnA</i>)	7		37		2C
207-a	<i>S. suis</i>	5'-nucleotidase (<i>sntC</i>)		11		26	3
207-b	<i>S. suis</i>	5'-nucleotidase (<i>sntC</i>)		7		15	3
X TRANSCRIPTION							
1 Degradation of RNA							
37	<i>S. pneumoniae</i>	Endoribonuclease L-PSP (<i>sp1567</i>)	3		28		2A, 2B
115**	<i>S. pneumoniae</i>	Polyribonucleotide nucleotidyltransferase (<i>pnpA</i>)	6		13		2C
2 RNA synthesis, modification, and DNA transcription							
6	<i>S. pyogenes</i>	Transcription termination-antitermination factor (<i>nusA</i>)	13		39		2B
15	<i>S. pyogenes</i>	DNA-Directed RNA polymerase alpha subunit (<i>rpoA</i>)	18		58		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
35-a	<i>S. pyogenes</i>	Transcription elongation factor (<i>greA</i>)	7		63		2B
35-b	<i>S. pyogenes</i>	Transcription elongation factor (<i>greA</i>)	10		64		2B
199	<i>S. pyogenes</i>	Transcription elongation factor (<i>greA</i>)		5		30	3
XI TRANSLATION							
1 Aminoacyl tRNA synthetases							
59	<i>S. pneumoniae</i>	Arginyl-tRNA synthetase (<i>argS</i>)	17		36		2C
72-a	<i>S. pyogenes</i>	Phenylalanyl-tRNA synthetase (alpha subunit) (<i>pheS</i>)	7		24		2C
72-b	<i>S. pyogenes</i>	Phenylalanyl-tRNA synthetase (alpha subunit) (<i>pheS</i>)	6		22		2C
88-a	<i>S. pneumoniae</i>	Tyrosyl-tRNA synthetase 1 (<i>tyrS</i>)	8		28		2C
88-b	<i>S. pneumoniae</i>	Tyrosyl-tRNA synthetase 1 (<i>tyrS</i>)	8		22		2C
112-a	<i>S. pneumoniae</i>	Glutamyl tRNA-gln amido-transferase subunit B (<i>gatB</i>)	12		34		2B
112-b	<i>S. pneumoniae</i>	Glutamyl tRNA-gln amido-transferase subunit B (<i>gatB</i>)	10		31		2B
116-a	<i>S. pyogenes</i>	Glutamyl tRNA-gln amido-transferase subunit A (<i>gatA</i>)	15		36		2B
116-b	<i>S. pyogenes</i>	Glutamyl tRNA-gln amido-transferase subunit A (<i>gatA</i>)	10		28		2B
117-a	<i>S. pyogenes</i>	Glutamyl-tRNA synthetase (<i>gltX</i>)	10		22		2B
117-b	<i>S. pyogenes</i>	Glutamyl-tRNA synthetase (<i>gltX</i>)	17	14	42	37	2B, 3
118-a	<i>S. pyogenes</i>	Threonyl-tRNA synthetase 1 (<i>thrS</i>)	12		23		2B
118-b	<i>S. pyogenes</i>	Threonyl-tRNA synthetase 1 (<i>thrS</i>)	11		22		2B
137	<i>S. pneumoniae</i>	Methionyl-tRNA synthetase (<i>metS</i>)	10		20		2B
139-a	<i>S. pneumoniae</i>	Lysyl-tRNA synthetase (<i>lysS</i>)	13		29		2B
139-b	<i>S. pneumoniae</i>	Lysyl-tRNA synthetase (<i>lysS</i>)	13		29		2B
200	<i>S. pyogenes</i>	Glutamyl tRNA-gln amido-transferase subunit C (<i>gatC</i>)		6		58	3
2 Degradation of proteins, peptides and glycoproteins							
48-a	<i>S. thermophilus</i>	Aminopeptidase C (<i>pepC</i>)	9		26		2B
48-b	<i>S. thermophilus</i>	Aminopeptidase C (<i>pepC</i>)	11		34		2B
145	<i>S. pyogenes</i>	ATP-Dependent clp protease proteolytic subunit (<i>clpP</i>)	3	5	18	19	2B, 3
182	<i>S. pyogenes</i>	Protease (<i>spy0591</i>)		8		29	3
3 Protein modifications							
128-a	<i>S. pneumoniae</i>	Polypeptide deformylase (<i>def</i>)	6		43		2C
128-b*	<i>S. pneumoniae</i>	Polypeptide deformylase (<i>def</i>)	3		21		2C
203**	<i>S. pneumoniae</i>	COF family protein/peptidyl-prolyl <i>cis-trans</i> isomerase, cyclophilin type (<i>sp1538</i>)		4		11	3
4 Ribosomal proteins: synthesis and modifications							
10-a	<i>S. pyogenes</i>	Ribosomal protein S1-like DNA-binding protein (<i>spy0913</i>)	7		32		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
10-b	<i>S. pyogenes</i>	Ribosomal protein S1-like DNA-binding protein (<i>spy0913</i>)	15	9	44	23	2B, 3
10-c	<i>S. pyogenes</i>	Ribosomal protein S1-like DNA-binding protein (<i>spy0913</i>)	12	13	35	33	2B, 3
31-a	<i>S. pneumoniae</i>	30S Ribosomal protein S2 (<i>rpsB</i>)	7		29		2B
31-b	<i>S. pneumoniae</i>	30S Ribosomal protein S2 (<i>rpsB</i>)	8		34		2B
31-c	<i>S. pneumoniae</i>	30S Ribosomal protein S2 (<i>rpsB</i>)	8		35		2B
39-a	<i>S. pneumoniae</i>	30S Ribosomal protein S6 (<i>rpsF</i>)	6	5	74	67	2B, 3
39-b	<i>S. pneumoniae</i>	30S Ribosomal protein S6 (<i>rpsF</i>)	4		67		2A, 2B
97-a	<i>S. pyogenes</i>	50S Ribosomal protein L1 (<i>rplA</i>)	6		33		2D
97-b	<i>S. pyogenes</i>	50S Ribosomal protein L1 (<i>rplA</i>)	5		31		2D
98	<i>S. pneumoniae</i>	30S Ribosomal protein S3 (<i>rpsC</i>)	6		41		2D
99	<i>S. pneumoniae</i>	50S Ribosomal protein L6 (<i>rplF</i>)	5		29		2D
100	<i>S. pyogenes</i>	50S Ribosomal protein L5 (<i>rplE</i>)	9		58		2D
103	<i>S. pneumoniae</i>	50S Ribosomal protein L31 type B (<i>rpmE2</i>)	6		67		2D
104	<i>S. pyogenes</i>	30S Ribosomal protein S8 (<i>rpsH</i>)	5		52		2D
106	<i>S. pyogenes</i>	50S Ribosomal protein L21 (<i>rplU</i>)	3		49		2D
108	<i>S. pneumoniae</i>	50S Ribosomal protein L13 (<i>rplM</i>)	3		39		2D
109	<i>S. pyogenes</i>	50S Ribosomal protein L3 (<i>rplC</i>)	3		22		2D
123	<i>S. pneumoniae</i>	50S Ribosomal protein L10 (<i>rplJ</i>)		7		39	3
134	<i>S. pneumoniae</i>	50S Ribosomal protein L17 (<i>rplQ</i>)	3		42		2D
156	<i>S. pyogenes</i>	50S Ribosomal protein L9 (<i>rplI</i>)	4		19		2D
201-a	<i>S. pyogenes</i>	50S Ribosomal protein L7/L12 (<i>rplL</i>)	6	7	62	64	2A, 3
201-b	<i>S. pyogenes</i>	50S Ribosomal protein L7/L12 (<i>rplL</i>)	3		45		2A
201-c	<i>S. pyogenes</i>	50S Ribosomal protein L7/L.12 (<i>rplL</i>)	3		45		2A
201-d	<i>S. pyogenes</i>	50S Ribosomal protein L7/L12 (<i>rplL</i>)	4		48		2A
5 Translation factors							
2-a	<i>S. pneumoniae</i>	Translation elongation factor G (<i>fusA</i>)	13	14	26	26	2B, 3
2-b	<i>S. pneumoniae</i>	Translation elongation factor G (<i>fusA</i>)	21	7	38	13	2B, 3
2-c	<i>S. pneumoniae</i>	Translation elongation factor G (<i>fusA</i>)	12	19	24	33	2B, 3
9-a	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	12		35		2A, 2B
9-b	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	13	16	43	47	2A, 2B, 3
9-c	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	11		29		2B
9-d	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	17		51		2B
9-e*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	8		20		2B
9-f*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	5		15		2A, 2B
9-g***	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	8		18		2A, 2B
9-h**	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	7		22		2A, 2B
9-i*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	11		44		2B
9-j**	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	5		15		2B
9-k***	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	12		40		2B
9-l*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	8		28		2B
9-m*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	4		11		2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
9-n**	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	6		17		2B
9-o***	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	7		21		2C
9-p*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	7		17		2D
9-q	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)		10		31	3
9-r*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	5		17		2A
9-s*	<i>S. oralis</i>	Translation elongation factor Tu (<i>tuf</i>)	4		12		2A
17-a	<i>S. pneumoniae</i>	Translation elongation factor TS (<i>tsf</i>)	6	14	30	58	2A, 2B, 3
17-b	<i>S. pneumoniae</i>	Translation elongation factor TS (<i>tsf</i>)	20	14	78	56	2B, 3
17-c**	<i>S. pneumoniae</i>	Translation elongation factor TS (<i>tsf</i>)	5		22		2B
22-a	<i>S. pyogenes</i>	Peptide chain release factor 1 (<i>prfA</i>)	7		27		2B
22-b***	<i>S. pyogenes</i>	Peptide chain release factor 1 (<i>prfA</i>)	3		8		2C
26-a	<i>S. pneumoniae</i>	Translation elongation factor P (<i>sp0435</i>)	5	5	42	31	2A, 2B, 3
26-b	<i>S. pneumoniae</i>	Translation elongation factor P (<i>sp0435</i>)		4		26	3
79	<i>S. pneumoniae</i>	Ribosome recycling factor (<i>ffr</i>)	9	4	51	20	2C, 3
XII TRANSPORT and BINDING PROTEINS							
1 Amino acids, peptides and amines							
65	<i>S. pneumoniae</i>	ABC transporter, ATP-binding protein (<i>sp0786</i>)	3		34		2A
81	<i>S. pneumoniae</i>	Branched-chain amino acid ABC transporter, ATP-binding protein (<i>sp0753</i>)	7		36		2C
84	<i>S. pneumoniae</i>	Amino acid ABC transporter, ATP-binding protein (<i>yjgE</i>)	6		30		2C
86	<i>S. pneumoniae</i>	Amino acid ABC transporter, ATP-binding protein (<i>sp0610</i>)	9	5	57	25	2C, 3
144	<i>S. pyogenes</i>	Amino acid ABC transporter, ATP-binding protein (<i>spy1506</i>)	6		37		2B
159	<i>S. pneumoniae</i>	ABC transporter ATP-binding protein (<i>sp0867</i>)	9	5	48	27	2A, 2B, 3
169	<i>S. pneumoniae</i>	Amino acid ABC transporter, ATP-binding protein (<i>sp0452</i>)	5		38		2D
205***	<i>S. pneumoniae</i>	ABC transporter substrate-binding protein-branched chain amino acid transport (<i>livJ</i>)		7		21	3
2 Carbohydrates							
73-a	<i>S. mutans</i>	Multiple sugar-binding transport ATP-binding protein (<i>msmK</i>)	7		25		2C
73-b	<i>S. mutans</i>	Multiple sugar-binding transport ATP-binding protein (<i>msmK</i>)	6		24		2C
77	<i>S. pyogenes</i>	Multiple sugar-binding ABC transport system, ATP-binding protein (<i>msmK</i>)	6		22		2C, 2D
3 Cations							
23-a	<i>S. mutans</i>	Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	9	7	39	33	2B, 3
23-b	<i>S. mutans</i>	Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	8		36		2A, 2B

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
23-c**	<i>S. mutans</i>	Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	4		16		2B
89	<i>S. pneumoniae</i>	ABC transporter ATP-binding protein-manganese transport (<i>psaB</i>)	8		42		2C
4 Phosphoenolpyruvate phosphotransferase							
44-a	<i>S. salivarius</i>	Mannose-specific phospho- transferase system component IIAB (<i>manL</i>)	8	14	33	47	2B, 3
44-b	<i>S. salivarius</i>	Mannose-specific phospho- transferase system component IIAB (<i>manL</i>)	11		45		2A, 2B
44-c*	<i>S. salivarius</i>	Mannose-specific phospho- transferase system component IIAB (<i>manL</i>)	6		23		2A
5 General							
159	<i>S. pneumoniae</i>	ABC transporter ATP-binding protein (<i>sp0867</i>)	9	9	44	47	2A, 2B, 3
93-a	<i>S. mutans</i>	SloA (<i>sloA</i>)	9		50		2C, 2D
93-b	<i>S. mutans</i>	SloA (<i>sloA</i>)	7		38		2D
172	<i>L. innocua</i>	OpuCA protein (<i>opuCA</i>)	9		30		2D
XIII OTHER CATEGORIES							
1 Adaption and atypical conditions							
3-a	<i>S. pneumoniae</i>	ATP-Dependent protease ATP-binding subunit (<i>clpL</i>)	20		37		2B
3-b	<i>S. pneumoniae</i>	ATP-Dependent protease ATP-binding subunit (<i>clpL</i>)	28		55		2B
19	<i>S. pyogenes</i>	ATP-Dependent protease (<i>clpE</i>)	9		15		2B
52	<i>S. mutans</i>	Responder protein (<i>covR</i>)	5	8	39	32	2B, 3
163-a	<i>S. mutans</i>	DPR protein (<i>dpr</i>)	8	7	45	45	2A, 2B, 3
163-b	<i>S. mutans</i>	DPR protein (<i>dpr</i>)	10		52		2A, 2B
163-c	<i>S. mutans</i>	DPR protein (<i>dpr</i>)	6		35		2A
163-d	<i>S. mutans</i>	DPR protein (<i>dpr</i>)	5		36		2A
167	<i>S. pyogenes</i>	Alkaline-shock protein (<i>asp</i>)	3		26		2B
176	<i>S. pyogenes</i>	HSP-70 co-factor (<i>grpE</i>)	5		38		2A
178-a	<i>S. mutans</i>	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		7		18	3
178-b	<i>S. mutans</i>	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		9		24	3
178-c	<i>S. mutans</i>	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		6		18	3
178-d	<i>S. mutans</i>	General stress protein (glucan-binding protein b) (<i>gsp-781</i>)		8		20	3
180	<i>S. algalactiae</i>	Group B streptococcus secreted protein (<i>bsp</i>)		10		23	3
186	<i>S. equisimilis</i>	Glyceraldehyde-3-phosphatedehydrogenase [plasminogen-binding protein] (<i>gpd</i>)		14		59	3
192	<i>S. gordonii</i>	Extracellular glyceraldehyde-3-phosphate dehydrogenase[fragment]		9		27	3
193	<i>S. mutans</i>	LemA like protein (<i>lemA</i>)	6		36		2A
194	<i>S. mutans</i>	GrpE protein [HSP-70 co-factor](<i>grpE</i>)		4		28	3

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
2 Phage related functions							
105	<i>S. pyogenes</i>	Single strand binding protein-phage associated (<i>spy1830</i>)	5	6	39	45	2B, 3
3 Transposon related functions							
173	<i>S. pneumoniae</i>	Transposase from transposonTN1545(<i>int</i>)	8		26		2D
4 No function assigned							
27	<i>S. coelicolor</i>	Hypothetical conserved protein (<i>sck15.04C</i>)	4		47		2D
33-a	<i>B. halodurans</i>	BH0339 (<i>bh0339</i>)	5		24		2B
33-b*	<i>B. halodurans</i>	BH0339 (<i>bh0339</i>)	3		14		2B
38-a	<i>S. pyogenes</i>	Hypothetical protein SPY1780 (<i>spy1780</i>)	8		81		2A, 2B
38-b	<i>S. pyogenes</i>	Hypothetical protein SPY1780 (<i>spy1780</i>)	4		35		2B
40-a	<i>S. pyogenes</i>	Hypothetical protein spy1564 (<i>spy1564</i>)	10		39		2B
40-b	<i>S. pyogenes</i>	Hypothetical protein spy1564 (<i>spy1564</i>)	11		47		2B
40-c	<i>S. pyogenes</i>	Hypothetical protein spy1564 (<i>spy1564</i>)	14		54		2B
40-d	<i>S. pyogenes</i>	Hypothetical protein spy1564 (<i>spy1564</i>)	10		38		2B
40-e	<i>S. pyogenes</i>	Hypothetical protein spy1564 (<i>spy1564</i>)	15		60		2B
40-f	<i>S. pyogenes</i>	Hypothetical protein spy1564 (<i>spy1564</i>)	8		33		2B
54	<i>S. mitis</i>	Hypothetical 19.2 kDa protein [fragment]	3		27		2B
64	<i>S. pyogenes</i>	Hypothetical protein spy1020 (<i>spy1020</i>)	6		12		2C
80	<i>S. pyogenes</i>	Hypothetical protein spy1518 (<i>spy1518</i>)	4		27		2C
82	<i>S. pneumoniae</i>	Hypothetical conserved protein spr1019 (<i>spr1019</i>)	8		48		2C
83	<i>S. pyogenes</i>	Hypothetical protein spy0652 (<i>spy0652</i>)	8		31		2C
90-a	<i>S. pyogenes</i>	Conserved protein-function unknown (<i>spy1613</i>)	10		73		2C, 2D
90-b	<i>S. pyogenes</i>	Conserved protein-function unknown (<i>spy1613</i>)	8		71		2C
90-c	<i>S. pyogenes</i>	Conserved protein-function unknown (<i>spy1613</i>)	8		71		2C
90-d	<i>S. pyogenes</i>	Conserved protein-function unknown (<i>spy1613</i>)	7		57		2C
91	<i>S. pneumoniae</i>	Hypothetical conserved protein spr0664 (<i>spr0664</i>)	9		62		2C, 2D
94**	<i>S. pyogenes</i>	Hypothetical protein spy1565 (<i>spy1565</i>)	5		12		2C, 2D
95	<i>L. innocua</i>	LIN2189 protein (<i>lin2189</i>)	6		33		2D
102	<i>S. pyogenes</i>	Hypothetical protein spy1511 (<i>spy1511</i>)	3		49		2D
121	<i>S. pyogenes</i>	Conserved hypothetical protein (<i>spyM18_0873</i>)	5		66		2A
129-a	<i>S. pyogenes</i>	Hypothetical 8.7 kDa protein (<i>spyM18_1526</i>)	3		48		2A
129-b	<i>S. pyogenes</i>	Hypothetical 8.7 kDa protein (<i>spyM18_1526</i>)	4		62		2A
146	<i>S. pyogenes</i>	Hypothetical protein spy0260 (<i>spy0260</i>)	4		25		2C
148	<i>S. mutans</i> LT11	Hypothetical 17.4 kDa protein	5		29		2C
149	<i>S. pyogenes</i>	Hypothetical protein spy1249 (<i>spy1249</i>)	4		38		2C
153	<i>S. pneumoniae</i>	Jag protein, putative (<i>sp2040</i>)	8		26		2C

Table 1. Continued

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function (gene)	Peptides matched ^{c)}		% Cover-age ^{d)}		Figure ^{e)}
			C	EC	C	EC	
158	<i>S. pyogenes</i>	Hypothetical protein spy1959 (<i>spy1959</i>)	5	6	41	45	2A, 2B, 3
164	<i>S. pyogenes</i>	Hypothetical protein spy2114 (<i>spy2114</i>)	3		33		2A, 2B
166	<i>S. pneumoniae</i>	Hypothetical 12.9 kDa protein (<i>spr0688</i>)	5		61		2B
171	<i>S. thermophilus</i>	Hypothetical 19.4 kDa protein	5		62		2D
191	<i>S. pyogenes</i>	Hypothetical protein spy1875 (<i>spy1875</i>)	4		56		2A
198	<i>S. pyogenes</i>	Hypothetical protein spy2065 (<i>spy2065</i>)		4		24	3
204	<i>S. pyogenes</i>	Hypothetical protein spy0316 (<i>spy0316</i>)		6		24	3
212	<i>S. mutans</i>	Hypothetical 31.5 kDa protein		8		32	3
213	<i>S. pneumoniae</i>	Hypothetical protein sp1027 (<i>sp1027</i>)		7		30	3
221	<i>L. lactis</i>	Hypothetical protein yfhK (<i>yfhK</i>)	3		24		2B

- a) Spot ID is the protein spot number on Fig. 2 and/or Fig. 3. *, denotes peptide matching the N-terminal region; **, denotes peptide matching the C-terminal region; ***, denotes peptide matching an internal region of the putative homologue.
- b) The bacterial species with the highest protein sequence similarity to the corresponding translated ORF from the *S. mutans* UA159 genome.
- c) The number of identified peptides from cellular (C) or extracellular (EC) proteins matching a given translated ORF from the *S. mutans* UA159 genome.
- d) The percentage coverage of the peptides over the matching translated ORF from the *S. mutans* UA159 genome.
- e) Figure(s) containing the corresponding protein spot.

Table 2. Trypsin digested peptides from *S. mutans* LT11 proteins sequenced and identified with ESI-MS/MS

Spot ID ^{a)}	Species with homologous protein ^{b)}	Putative function	Subcellular location	Sequence ^{c)}
58	<i>B. subtilis</i>	YdcP protein	cellular/extracellular	SSAGG(L/I)(L/I)TVTVPEAK
197	<i>B. subtilis</i>	TopI homolog	extracellular	DQG(L/I)N(L/I)AER
202-a	<i>S. mutans</i>	Phosphocarrier protein, HPr (histidine-containing protein)	extracellular	1. FASD(L/I)T(L/I)DYK 2. PAT(L/I)(L/I)VQTASK
202-b	<i>S. mutans</i>	Phosphocarrier protein, HPr (histidine-containing protein)	extracellular	1. FASD(L/I)T(L/I)DYK 2. PAT(L/I)(L/I)VQTASK
44-d	<i>S. salivarius</i>	Mannose-specific phosphotransferase system component IIAB (ManL)	cellular	(L/I)PF(L/I)(L/I)QAYTER

- a) Spot ID is the protein spot number on Fig. 2 and/or Fig. 3
- b) The bacterial species with the highest protein sequence similarity
- c) The residues leucine (L) and isoleucine (I) are of the same mass and are indistinguishable with ESI-MS/MS. The underlined residue highlighted in bold is the residue matching that in the homologous protein.

Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance

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Two-dimensional gel electrophoretic analysis of the proteome of *Streptococcus mutans* grown at a steady state in a glucose-limited anaerobic continuous culture revealed a number of proteins that were differentially expressed when the growth pH was lowered from pH 7.0 to pH 5.0. Changes in the expression of metabolic proteins were generally limited to three biochemical pathways: glycolysis, alternative acid production and branched-chain amino acid biosynthesis. The relative level of expression of protein spots representing all of the enzymes associated with the Embden–Meyerhof–Parnas pathway, and all but one of the enzymes involved in the major alternative acid fermentation pathways of *S. mutans*, was identified and measured. Proteome data, in conjunction with end-product and cell-yield analyses, were consistent with a phenotypic change that allowed *S. mutans* to proliferate at low pH by expending energy to extrude excess H⁺ from the cell, while minimizing the detrimental effects that result from the uncoupling of carbon flux from catabolism and the consequent imbalance in NADH and pyruvate production. The changes in enzyme levels were consistent with a reduction in the formation of the strongest acid, formic acid, which was a consequence of the diversion of pyruvate to both lactate and branched-chain amino acid production when *S. mutans* was cultivated in an acidic environment.

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INTRODUCTION

The initiation of dental caries is clinically associated with acidogenic and aciduric bacteria that secrete and tolerate the organic acidic fermentation by-products of carbohydrate metabolism. By their demineralizing action upon tooth enamel, these organic acids both initiate and contribute to the aetiology of the disease. Over the past 50 years, studies of dental caries have focussed on the oral streptococci, particularly *Streptococcus mutans*, which is now accepted to be associated with the initiation of various forms of the disease (Hamada & Slade, 1980; Harper & Loesche, 1984; Loesche, 1986; van Houte, 1994; van Ruyven *et al.*, 2000).

Much research has been devoted to studying streptococcal carbohydrate metabolism, particularly the biochemical and physiological adaptations that allow *S. mutans* to produce acids and to survive at low pH in the oral cavity. Historically, this research has employed a reductionist approach to study biochemical processes such as the allosteric regulation of enzymes and the alteration in fluxes of metabolites (Iwami

& Yamada, 1980; Hamilton, 1984; Yamada, 1987; Carlsson & Hamilton, 1996; Quivey *et al.*, 2001). A number of recent reports, however, have made use of the more holistic strategy enabled by proteome analysis to re-evaluate the mechanism of aciduricity in *S. mutans*. For example, an analysis by two-dimensional electrophoresis (2-DGE) of ¹⁴C-labelled cellular proteins revealed 64 proteins that were up-regulated, and 49 that were down-regulated, when cells were shocked from pH 7.5 to pH 5.0. The identities of these proteins remain to be determined (Svensäter *et al.*, 2000). In a related study, 18 proteins were up-regulated and 12 down-regulated when a comparison was made between the 2-DGE proteomes of *S. mutans* grown in batch culture, without pH control, from starting pH values of 7.0 and 5.2. Of the 27 proteins identified by mass spectroscopic analysis, 13 were involved in sugar transport and central metabolism (Wilkins *et al.*, 2002). In this latter report, *S. mutans* was harvested and analysed at mid-exponential phase, at which stage the pH values of the two cultures had fallen to 6.2 and 4.7, respectively. Significantly dissimilar generation times were obtained, 1.0 h and 6.6 h, at the two pHs (Wilkins *et al.*, 2002). This variation in pH in batch culture makes an understanding of the observed changes in the proteome difficult to evaluate solely in relation to pH, since cells harvested from batch cultures have been continuously responding to an ever-changing external environment. In contrast, the continuous-culture method used in this study has the advantage of allowing cells to be sampled under

Abbreviations: 2-DGE, two-dimensional gel electrophoresis; ASB-14, amidosulfobetaine-14; *D*, dilution rate; DE, differential expression (values); IPG, immobilized pH gradient; IPS, intracellular polysaccharide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PEP-PTS, phosphoenolpyruvate:glucose phosphotransferase system; PMM, peptide mass mapping; *Y*_{ATP}, ATP yield, dry weight cells per mol ATP; *Y*_{Glc}, cell yield, dry weight cells per mol glucose.

strictly defined conditions at steady state, thus enabling true comparative proteome analysis when individual parameters are altered.

In the current study, the proteome of *S. mutans*, growing at steady state at pH 7.0 in a defined artificial salivary medium, has been compared with that of cells grown at pH 5.0. This approach has been chosen to mimic the conditions in the dental plaque of a healthy individual in comparison with those of a more susceptible host. While, at one level, this analysis has highlighted the extent of phenotypic adaptation during acid tolerance, at another it has shown that changes in the level of expression of proteins appear to be limited to key metabolic pathways; notably, glycolysis, acid production, and the synthesis of branched-chain amino acids. In contrast to previous studies, where none (Svensäter *et al.*, 2000), or fewer than 18% (Wilkins *et al.*, 2002) of the proteins in these pathways were detected, this study identified 83% of the enzymes in these three biochemical pathways. The data were consistent with the hypothesis that *S. mutans* adapts to an acidic environment by minimizing H^+ production, using a series of different strategies. This was the case, despite the drain on ATP required for H^+ extrusion and the resultant uncoupling of carbon utilization from anabolic processes.

METHODS

Bacterial strain and growth conditions. Continuous cultures of *S. mutans* LT11 (Tao *et al.*, 1993) were grown under anaerobic conditions at a dilution rate (D) of $0.100 \pm 0.001 \text{ h}^{-1}$, at pH 7.0 ± 0.1 or at pH 5.0 ± 0.1 , with glucose as the limiting nutrient, as previously described (Jacques *et al.*, 1979). DMM medium was used, without mucin, but modified to include adenine, guanine and uracil, at $20 \mu\text{g ml}^{-1}$, 15 mM KH_2PO_4 and 15 mM K_2HPO_4 (Sissons *et al.*, 1991). Analyses showed that *S. mutans* was glucose limited at $D=0.1 \text{ h}^{-1}$, both at pH 7.0 and at pH 5.0, since 99.8% and 99.9%, respectively, of the glucose in the medium was utilized, while all of the added amino acids remained in excess (mean utilization, 75% at pH 7.0 and 72% at pH 5.0).

Preparation of cellular proteins. When steady state had been achieved, the bacterial contents of the culture vessel were harvested, washed and lyophilized, before aliquots (10 mg dry wt) of cells were treated with mutanolysin (Len *et al.*, 2003). Proteins that were to be separated on acidic immobilized pH gradient (IPG) strips (pH 4.0–6.7) were extracted as previously described (Len *et al.*, 2003), except that 1% (w/v) amidosulfo betaine-14 (ASB-14) and 65 mM DTT were added to produce a modified solubilizing solution for 2-DGE. While the addition of these reagents increased the total number of protein spots that could be readily discerned on 2-DGE gels, their inclusion selectively inhibited the extraction or subsequent separation of a small number of weakly expressed proteins that had been previously visualized and/or identified on 2-DGE gels (Len *et al.*, 2003).

Proteins that were to be separated on basic IPG strips (pH 6–11) were obtained from the mutanolysin-treated cells by a two-fraction solubilization procedure. Following centrifugation of the cell lysate (12 000 g, 4 °C, 10 min), the cell pellet was stored at –20 °C, and proteins in the supernatant were precipitated overnight at –20 °C with 15% (w/v) trichloroacetic acid. Following centrifugation (12 000 g, 4 °C, 10 min) and two washes in methanol, the precipitated proteins

were solubilized in 300 μl of a 1:1 mixture of modified solubilization solution (without ASB-14) and Cellular and Organelle Membrane Solubilizing Reagent (Sigma-Aldrich), containing 1% (v/v) Triton X-100 and 2 mM tributylphosphine. The frozen cell pellet was thawed and then resuspended by sonication (Branson Ultrasonics; 50 W, $10 \times 10 \text{ s}$, with cooling on ice between each burst) in 700 μl of the same solubilization solution. Exonuclease III (150 U) was added, and the suspension incubated at room temperature (20–22 °C) for 15 min to degrade any DNA. The two cellular fractions were then combined and centrifuged at room temperature (12 000 g, 20–22 °C, 10 min). Prior to IEF, 100 μl iodoacetamide (500 mM) was added and the mixture incubated at room temperature (20–22 °C) for 2 h.

2-DGE. In order to separate acidic proteins by 2-DGE, the equivalent of the amount of cellular proteins extracted from 0.75 mg dry cell weight was dissolved in 150 μl modified 2-DGE-solubilizing solution, prior to cup-loading onto narrow-range (pH 4.0–5.0, 4.5–5.5 and 5.5–6.7) IPG strips (Amersham Biosciences), pre-swollen with 350 μl of the same modified 2-DGE-solubilizing solution. For basic cellular proteins, IPG strips (pH 6–11; Amersham Biosciences) were first pre-swollen with modified 2-DGE-solubilizing solution, without ASB-14 but containing 10% (v/v) 2-propanol. Cellular proteins extracted from the equivalent of 1 mg dry cell weight were then cup-loaded onto IPG strips. This modification resulted in better 2-DGE resolution of basic proteins.

Proteins were focused using a Multiphor II electrophoresis system (Amersham Biosciences) at 20 °C. Proteins were focussed on narrow-range IPG strips for a total of 141.5 kVh (100 V for 7 h, followed by 300 V for 6 h, 1000 V for 2 h, 2500 V for 2 h, 3500 V for 2 h and 5000 V for 25 h) and on broad-range IPG strips for a total of 79.8 kVh (100 V for 2 h, followed by 300 V for 2 h, 1000 V for 2 h, 2500 V for 2 h, 3500 V for 12 h and 5000 V for 6 h). Following separation in the second dimension on 10–18% T gradient SDS-PAGE, the gels were stained with Sypro Ruby for image analysis, before being ‘double stained’ with Coomassie blue CBB G-250 for spot excision, tryptic digestion and mass spectroscopic analysis (Len *et al.*, 2003). A high level of reproducibility of protein spots on Sypro Ruby-stained 2-DGE gels was observed between samples obtained from the the same growth pH (data not shown).

The density/volume, or differential expression (DE) value (arbitrary units), of each Sypro Ruby-stained protein spot was determined using the software package z3 (Compugen). In this study, the main source of error associated with this form of quantification was the reproducibility of the 2-DGE displays themselves, since biological variation was minimized through the use of a chemostat to culture the bacteria. That the 2-DGE displays were the main source of error was confirmed by comparing the DE values of 25 randomly chosen protein spots, selected from 2-DGE displays separated on broad-range IPG strips (pH 4.0–7.0). Equivalent protein spots from triplicate samples from each of three repeat continuous cultures were analysed. The data confirmed that 2-DGE was the main source of error in determining DE values. As a consequence, triplicate experimental samples from cells grown at each pH were used for all 2-DGE analyses, and the increase in the level of expression of a protein spot was based on the change in mean DE value.

Protein isoforms. The term ‘isoform’ is used here to describe the multiple charged forms of a protein that exist on 2-DGE gels, where the mean observed M_r for each form calculated from the second (SDS-PAGE) dimension deviates by 5% or less, and where there is no evidence from peptide-mass mapping of any form of truncation or degradation.

Mass spectrometry. Tryptic in-gel digestion, concentration and desalting of low-copy-number proteins, and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopic

analysis were carried out (as previously described) on each protein spot that was differentially or uniquely expressed at pH 7.0 or pH 5.0, or that formed part of the metabolic pathways of interest (Len *et al.*, 2003). Mass spectroscopic analysis of protein spots from each of the replicate 2-DGE gels, at both growth pHs, was used to confirm the identity of the proteins that had been matched by the z3 software.

Protein identification. Peptide mass mapping (PMM) analysis of proteins was undertaken as previously described, making use of the six contigs of the *S. mutans* UA159 genome downloaded on October 6, 2001 that were translated in all six reading frames (Len *et al.*, 2003). The original six contigs were used in this manner as some genes identified in these contigs were not present in the final annotated version.

Parameters for protein identification included a mass tolerance of 150 p.p.m. and a maximum of one missed cleavage per peptide, while taking into consideration methionine sulfoxide and cysteine acryl amide modifications. Matches were defined on the basis of the number of matching peptide masses and the total percentage sequence covered by the peptides. As a general rule, a minimum total sequence coverage of 25 % was taken to match a given translated ORF of a high- M_r protein with confidence, though coverage as high as 80 % was observed with many low- M_r proteins. All translated ORFs that matched PMM data were then used to query the annotated *S. mutans* genome at the Oral Pathogen Sequence Databases (<http://www.stdgen.lanl.gov/oragen>), using the local BLAST search facility to determine the gene identification number. All gene names used are those associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site. Theoretical M_r and pI were determined using MassLynx software version 3.4 (Micromass).

Amino acid analysis. The concentrations of free amino acids in the spent medium were analysed by pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, using a Waters AccQFluor reagent kit (Cohen & Michaud, 1993; Cohen & DeAntonis, 1994). Amino acid derivatives were separated and quantified by reversed-phase (C_{18}) HPLC, using a Waters AccQTag column (15 cm $\times$ 3.9 mm i.d.), according to the manufacturer's protocol (Cohen, 2001). HPLC was carried out with a Waters Alliance 2695 Separation Module, 474 Fluorescence Detector and 2487 Dual λ Absorbance Detector, in series. The control and analysis software was Waters Empower Pro Module. Tryptophan was analysed by UV detection, and all other amino acids by fluorescence detection.

Metabolite analyses. The concentrations of glucose, acetate, ethanol, formate and lactate present in the medium at steady state were measured enzymically with the appropriate detection kit (Roche), according to the manufacturer's instructions (Harty & Handley, 1988). The amount of accumulated intracellular polysaccharide (IPS) was determined by the potassium iodide method of DiPersio *et al.* (1974).

RESULTS AND DISCUSSION

A combination of steady-state continuous-culture technology and narrow-range IPG strips allowed the detection, on Sypro Ruby-stained 2-DGE gels, of 155 differentially expressed cellular protein spots upregulated more than 1.5-fold during acid-tolerant growth of *S. mutans* at pH 5.0. Of these, 123 were identified by MALDI-TOF analysis and 53 found to be associated with regulatory and/or stress-responsive pathways (Len *et al.*, 2004). The remaining 70 protein spots were associated with metabolism; the majority

were associated with glycolysis, alternative acid production and branched-chain amino acid synthesis (Table 1).

Glucose uptake and proton extrusion

At pH 7.0, *S. mutans* preferentially transports glucose by the Enzyme II complexes of high-affinity phosphoenolpyruvate:glucose phosphotransferase systems (PEP-PTS), capable of scavenging glucose molecules (Ellwood *et al.*, 1979). Two such systems exist in *S. mutans*: EII^{man}, which has been well characterized, and EII^{glc}, the existence of which is primarily based on physiological observations (Vadeboncoeur, 1984; Néron & Vadeboncoeur, 1987). However, continuous-culture studies suggest that *S. mutans* is completely devoid of EII^{man} and EII^{glc} activity at pH 5.0 (Vadeboncoeur *et al.*, 1991). At this acid pH, the bacterium preferentially utilizes a non-PTS glucose permease system (Hamilton & St Martin, 1982; Dashper & Reynolds, 1990; Buckley & Hamilton, 1994), while maintaining a more alkaline cytoplasm by increasing the level of its proton-translocating F_1F_0 -ATPase (Hamilton & Buckley, 1991; Cvitkovitch *et al.*, 1995). The recent annotation of the *S. mutans* genome, however, does not identify a gene encoding a specific glucose permease (Ajdić *et al.*, 2002).

MALDI-TOF analysis failed to identify any intrinsic membrane proteins associated with the uptake of glucose in *S. mutans*. The failure to detect denatured intrinsic hydrophobic membrane proteins in Gram-positive bacteria is a common problem with 2-DGE proteome analysis (Len *et al.*, 2003). Despite this limitation, two forms of the cytoplasmic component, EIIAB^{man} (ManL) of the EII^{man} PEP-PTS, were identified (Fig. 1, Table 1). Unfortunately, these two forms, of M_r 16 060 $\pm$ 80 and 14 940 $\pm$ 30, with pI 4.44 $\pm$ 0.05 and 4.46 $\pm$ 0.02, respectively ($n=6$), were C-terminally truncated. They represented only the EIIA portion of the protein, since peptide mass fingerprint coverage was limited to the EIIA region (data not shown). Although the EIIAB^{man} component of the EII^{man} PEP-PTS has been reported to be constitutively expressed in *S. mutans* (Vadeboncoeur, 1984), the truncated nature of the proteins observed in this study severely limited the interpretation of the observed 6.8-fold down-regulation of these proteins at pH 5.0. This limitation also applied to the question of whether the down-regulation reflected a reduction in the use of the glucose PEP-PTS under these acidic conditions (Fig. 1, Table 1).

In contrast, glucokinase (Glk), required for the ATP-dependent phosphorylation of any free glucose entering the cell by way of a permease, was up-regulated by 2.1-fold at pH 5.0. However, as glucokinase has a pH optimum of approximately 8.0 (Porter *et al.*, 1982), its relative activity and stability are affected by acidification of the cytoplasm at a growth pH of 5.0, where the internal pH would be less than 6.5 (Dashper & Reynolds, 1990; Hamilton, 1990; Iwami *et al.*, 1992). Since the optimal pH for glycolysis appears to be 7.0 (Dashper & Reynolds, 1992), the reduction in cytoplasmic pH most likely explains why glucokinase is a

Table 1. Differential expression of metabolic proteins by *S. mutans* grown at pH 7.0 or pH 5.0

Gene ID, gene names associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site (<http://www.stdgen.lanl.gov/oragen>).

Spot no.	Gene ID	Enzyme, EC no. and gene	2-DGE coordinates (pI/M _r)		DE value ¹	
			Theoretical	Observed ¹	pH 7.0	pH 5.0
1a	SMu1707	Mannose-specific phosphotransferase system component IIAB (EC 2.7.1.69) (<i>manL</i>)*	5.20/35 562	4.44 ± 0.06/16 060 ± 80	4 449 ± 667	661 ± 78
1b	SMu1707	Mannose-specific phosphotransferase system component IIAB (EC 2.7.1.69) (<i>manL</i>)*	5.20/35 562	4.46 ± 0.02/14 940 ± 30	3 879 ± 589	562 ± 47
2a	SMu1391	ATP synthase alpha chain (EC 3.6.3.14) (<i>atpA</i>)	5.00/49 350	4.87 ± 0.02/53 760 ± 180	807 ± 313	6 079 ± 172
2b	Smu1388	ATP synthase epsilon chain (EC 3.6.3.14) (<i>atpC</i>)	6.50/15 588	6.50/15 588†	1 363 ± 425	4 851 ± 412
3	SMu0493	Glucose kinase (EC 2.7.1.2) (<i>glk</i>)	4.90/33 635	4.90 ± 0.00/32 230 ± 200	1 898 ± 172	3 908 ± 367
4	SMu0277	Glucose-6-phosphate isomerase (EC 5.3.1.9) (<i>gpi</i>)	4.90/49 422	4.82 ± 0.02/49 310 ± 230	4 256 ± 387	8 480 ± 1 443
5a	SMu1090	6-Phosphofructokinase (EC 2.7.1.11) (<i>pfk</i>)	5.60/35 771	5.48 ± 0.02/36 350 ± 860	4 098 ± 330	5 452 ± 637
5b	SMu1090	6-Phosphofructokinase (EC 2.7.1.11) (<i>pfk</i>)	5.60/35 771	5.50 ± 0.00/36 070 ± 1 060	837 ± 39	639 ± 55
6a	SMu0088	Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) (<i>fbaA</i>)	5.30/32 996	5.00 ± 0.00/28 670 ± 140	1 275 ± 81	188 ± 11
6b	SMu0088	Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) (<i>fbaA</i>)	5.30/32 996	5.00 ± 0.00/29 190 ± 90	3 207 ± 167	5 390 ± 1 253
6c	SMu0088	Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) (<i>fbaA</i>)	5.30/32 996	5.00 ± 0.00/29 130 ± 70	12 527 ± 980	20 473 ± 2 375
7a	SMu0652	Triosephosphate isomerase (EC 5.3.1.1) (<i>tpiA</i>)	5.10/27 211	5.00 ± 0.00/24 190 ± 80	1 223 ± 218	1 164 ± 360
7b	SMu0652	Triosephosphate isomerase (EC 5.3.1.1) (<i>tpiA</i>)	5.10/27 211	5.00 ± 0.00/22 160 ± 570	1 295 ± 163	1 395 ± 36
7c	SMu0652	Triosephosphate isomerase (EC 5.3.1.1) (<i>tpiA</i>)	5.10/27 211	4.90 ± 0.00/21 920 ± 260	493 ± 33	379 ± 74
8a	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	5.38 ± 0.04/35 240 ± 310	5 550 ± 73	1 172 ± 138
8b	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	5.90 ± 0.00/36 590 ± 350	4 965\$	7 942\$
8c	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	6.00/36 068†	68 609\$	107 697\$
8d	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	5.50 ± 0.00/38 910 ± 850	12 390 ± 1 097	18 177 ± 6 495
8e	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)*	6.00/36 068	5.34 ± 0.03/28 850 ± 600	690 ± 168	539 ± 85
8f	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	5.50 ± 0.00/39 280 ± 760	1 316 ± 320	1 078 ± 211
8g	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	5.40 ± 0.00/39 590 ± 540	2 622 ± 433	1 879 ± 181
8h	SMu0325	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (<i>gapDH</i>)	6.00/36 068	6.10 ± 0.00/37 450 ± 190	7 275\$	12 491\$
9a	SMu0618	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) (<i>gapN</i>)	5.20/47 166	5.20 ± 0.00/55 680 ± 380	3 462 ± 65	6 646 ± 145
9b	SMu0618	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) (<i>gapN</i>)	5.20/47 166	5.20 ± 0.00/55 390 ± 300	1 672 ± 198	5 756 ± 459
10a	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)	5.50/43 436	5.30 ± 0.00/42 730 ± 460	12 222 ± 500	12 898 ± 1 189
10b	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)	5.50/43 436	5.20 ± 0.00/42 580 ± 320	6 669 ± 835	10 607 ± 1 321
10c	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)*	5.50/43 436	6.30 ± 0.00/26 100 ± 90	2 583 ± 83	4 478 ± 412
10d	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)	5.50/43 436	5.25 ± 0.02/44 570 ± 440	2 616 ± 824	1 511 ± 224
10e	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)*	5.50/43 436	6.60 ± 0.00/29 790 ± 50	3 645 ± 342	262 ± 153
10f	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)*	5.50/43 436	4.82 ± 0.02/38 640 ± 220	1 609 ± 52	798 ± 99
10g	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)*	5.50/43 436	4.90 ± 0.00/38 770 ± 260	1 564.33 ± 64	693 ± 96
10h	SMu0326	Phosphoglycerate kinase (EC 2.7.2.3) (<i>pgk</i>)*	5.50/43 436	4.80 ± 0.00/38 560 ± 150	1 507 ± 64	799 ± 99

Table 1. conti.

Spot no.	Gene ID	Enzyme, EC no. and gene	2-DGE coordinates (pI/M _r)		DE value	
			Theoretical	Observed	pH 7.0	pH 5.0
11a	SMu0543	Phosphoglyceromutase (EC 5.4.2.1) (<i>pgmY</i>)	5.20/26 050	5.02 ± 0.02/24 650 ± 50	1 039 ± 59	2 758 ± 73
11b	SMu0067	Phosphoglycerate mutase-like protein (EC 5.4.2.1) (<i>pgm</i>)	5.10/26 526	5.10 ± 0.00/25 390 ± 80	987 ± 108	0.0
12a	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)	4.70/46 858	4.70/46 858†	166 641 ± 20 849	12 293 ± 1423
12b	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)	4.70/46 858	4.60 ± 0.00/44 230 ± 250	3 937 ± 691	1 533 ± 580
12c	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)*	4.70/46 858	4.65 ± 0.02/28 240 ± 160	2 503 ± 104	636 ± 102
12d	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)	4.70/46 858	4.80 ± 0.00/42 190 ± 120	2 069 ± 302	0.0
12e	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)†	4.70/46 858	4.68 ± 0.02/26 990 ± 170	2 018 ± 173	655 ± 92
12f	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)†	4.70/46 858	4.62 ± 0.02/25 560 ± 140	1 991 ± 158	0.0
12g	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)	4.70/46 858	4.70 ± 0.00/41 070 ± 120	541 ± 238	2 006 ± 584
12h	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)†	4.70/46 858	4.70 ± 0.00/27 510 ± 190	2 018 ± 173	655 ± 91
12i	SMu1140	Enolase (EC 4.2.1.11) (<i>eno</i>)*	4.70/46 858	4.53 ± 0.02/9810 ± 100	2 093 ± 52	934 ± 32
13a	SMu1089	Pyruvate kinase (EC 2.7.1.40) (<i>pyk</i>)	5.10/54 367	5.10 ± 0.00/54 830 ± 150	3 565 ± 849	8 407 ± 2366
13b	SMu1089	Pyruvate kinase (EC 2.7.1.40) (<i>pyk</i>)	5.10/54 367	5.10 ± 0.00/54 430 ± 250	264 ± 34	1 874 ± 140
13c	SMu1089	Pyruvate kinase (EC 2.7.1.40) (<i>pyk</i>)	5.10/54 367	5.10/54 367‡	2 339 ± 230	5 457 ± 883
13d	SMu1089	Pyruvate kinase (EC 2.7.1.40) (<i>pyk</i>)	5.10/54 367	5.05 ± 0.02/54 410 ± 210	303 ± 60	2 354 ± 1037
14	SMu1398	Glycogen biosynthesis protein (glucose-1-phosphate adenylyltransferase) (EC 2.7.7.27) (<i>glgD</i>)	5.70/42 335	6.02 ± 0.02/43 170 ± 310	9 172\$	10 623\$
15	SMu0985	Putative phosphoglucomutase (EC 5.4.2.2) (<i>pgmA</i>)*	5.00/65 152	6.13 ± 0.02/27 100 ± 50	1 044 ± 226	0.0
16a	SMu1330	Glucose-1-phosphate thymidyltransferase (EC 2.7.7.24) (<i>rmlA</i>)	5.30/32 989	4.90 ± 0.00/28 560 ± 70	4 494 ± 453	1 851 ± 138
16b	SMu1330	Glucose-1-phosphate thymidyltransferase (EC 2.7.7.24) (<i>rmlA</i>)	5.30/32 989	5.00 ± 0.00/28 480 ± 60	1 136 ± 159	0.0
17	Smu1327	dTDP-glucose-4,6-dehydratase (EC 4.2.1.46) (<i>rmlB</i>)	5.80/39 268	—§	—§	—§
18	Smu1329	dTDP-4-keto-L-rhamnose reductase (EC 5.1.3.133) (<i>rmlC</i>)	4.80/17 206	—§	—§	—§
19	SMu0748	dTDP-4-keto-L-rhamnose reductase (EC 1.1.1.133) (<i>rmlD</i>)	4.80/32 398	5.00 ± 0.00/32 040 ± 90	2 429 ± 125	2 396 ± 405
20a	SMu1018	L-Lactate dehydrogenase (EC 1.1.1.27) (<i>ldh</i>)	5.10/35 245	5.00 ± 0.00/35 780 ± 70	1 818 ± 308	4 576 ± 305
20b	SMu1018	L-Lactate dehydrogenase (EC 1.1.1.27) (<i>ldh</i>)	5.10/35 245	5.10 ± 0.00/35 900 ± 40	6 442 ± 536	11 107 ± 840
21	SMu0115	Dihydrolipoamide S-acetyltransferase (EC 2.3.1.12) (<i>adhC</i>)†	5.60/48 674	5.30 ± 0.00/15 630 ± 180	2 159 ± 430	469 ± 80
22	SMu1297	Acetoin dehydrogenase E1 component (EC 1.2.4.1) (<i>acoB</i>)	4.70/37 158	4.80 ± 0.00/37 750 ± 200	1 504 ± 218	988 ± 349
23	SMu0363	Pyruvate formate-lyase (EC 2.3.1.54) (<i>pfl</i>)	5.20/87 600	—§	—§	—§
24	SMu0952	Phosphotransacetylase (EC 2.3.1.8) (<i>pta</i>)	4.80/36 369	4.90 ± 0.00/31 230 ± 130	578 ± 72	1 000 ± 36
25	SMu1207	Putative acetoin dehydrogenase (EC 1.1.1.5) (<i>butA</i>)	6.00/27 524	6.14 ± 0.02/28 010 ± 260	2 910 ± 87	91 ± 91
26a	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)	5.60/96 993	5.40 ± 0.00/89 870 ± 1 110	1 660 ± 370	285 ± 60
26b	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)	5.60/96 993	5.42 ± 0.18/89 590 ± 1 020	2 165 ± 260	286 ± 36
26c	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)	5.60/96 993	5.48 ± 0.017/89 400 ± 1 380	2 636 ± 337	365 ± 257
26d	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)	5.60/96 993	5.50 ± 0.00/89 520 ± 1 320	2 787 ± 502	1 340 ± 35
26e	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)	5.60/96 993	5.52 ± 0.02/88 900 ± 1 430	4 271 ± 563	1 816 ± 236
26f	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)	5.60/96 993	5.60 ± 0.00/89 000 ± 1 780	3 678 ± 125	1 321 ± 429
26g	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	5.40 ± 0.00/48 650 ± 120	756 ± 231	0.0

Table 1. cont.

Spot no.	Gene ID	Enzyme, EC no. and gene	2-DGE coordinates (pI/M _r)		DE value	
			Theoretical	Observed	pH 7.0	pH 5.0
26h	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	6.00 ± 0.00/63 870 ± 1 080	3 506 ± 898	0.0
26i	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	6.10 ± 0.00/65 090 ± 660	2 797 ± 186	0.0
26j	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	6.10 ± 0.00/57 190 ± 650	1 441 ± 133	0.0
26k	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	6.10 ± 0.00/55 660 ± 650	2 898 ± 206	53 ± 53
26l	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	6.00 ± 0.00/60 440 ± 220	2 325 ± 293	0.0
26m	SMu0131	Alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) (<i>adhE</i>)*	5.60/96 993	6.10 ± 0.00/40 860 ± 290	2 906 ± 340	0.0
27	Smu1186	Acetate kinase (EC 2.7.2.1) (<i>ackA</i>)	6.10/43 732	6.10 ± 0.00/43 650 ± 480	8 651 ± 477	4 084 ± 381
28a	SMu0211	Ketol-acid reductoisomerase (EC 1.1.1.86) (<i>ilvC</i>)	5.10/37 285	5.00 ± 0.00/37 260 ± 90	461 ± 97	1 818 ± 195
28b	SMu0211	Ketol-acid reductoisomerase (EC 1.1.1.86) (<i>ilvC</i>)	5.10/37 285	5.05 ± 0.02/37 210 ± 90	1 955 ± 372	11 075 ± 1 737
28c	SMu0211	Ketol-acid reductoisomerase (EC 1.1.1.86) (<i>ilvC</i>)	5.10/37 285	5.10/37 285‡	6 250 ± 389	16 212 ± 797
28d	SMu0211	Ketol-acid reductoisomerase (EC 1.1.1.86) (<i>ilvC</i>)	5.10/37 285	5.15 ± 0.02/37 820 ± 60	772 ± 16	2 731 ± 644
29	SMu1099	Branched-chain amino acid aminotransferase (EC 2.6.1.42) (<i>ilvE</i>)	5.10/37 732	5.10 ± 0.00/40 180 ± 130	3 133 ± 223	8 388 ± 536
30	SMu0329	Glutamine synthetase, type I (EC 6.3.1.2) (<i>glnA</i>)	5.20/49 955	5.20 ± 0.00/47 700 ± 250	401 ± 139	3 481 ± 146
31a	SMu0290	Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) (<i>gpdA</i>)	6.00/37 826	6.06 ± 0.02/35 430 ± 180	6 971 ± 455	2 588 ± 356
31b	SMu0290	Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) (<i>gpdA</i>)	6.00/37 826	6.00 ± 0.0/33 690 ± 80	10 202 ± 1832	5 385 ± 992
32	SMu0262	Probable transketolase (EC 2.2.1.1) (<i>tkt</i>)	5.00/71 076	5.00 ± 0.00/72 910 ± 490	0.0	1 145 ± 108
33	SMu1458	Lactoylglutathione lyase (EC 4.4.1.5) (<i>gloA</i>)	4.90/15 271	5.07 ± 0.02/17 980 ± 90	991 ± 111	2 599 ± 86

*N-terminus of protein.

†C-terminus of protein.

‡Part of the complement of random landmark proteins used to align gels and to calculate observed pI and M_r in accordance with the requirements of the z3 software.

§DE value obtained from a single 2-DGE gel. All other proteins were identified on triplicate gels.

||Mean ± SEM (n=3).

¶Previously detected by 2-DGE proteome analysis (Len *et al.*, 2003).

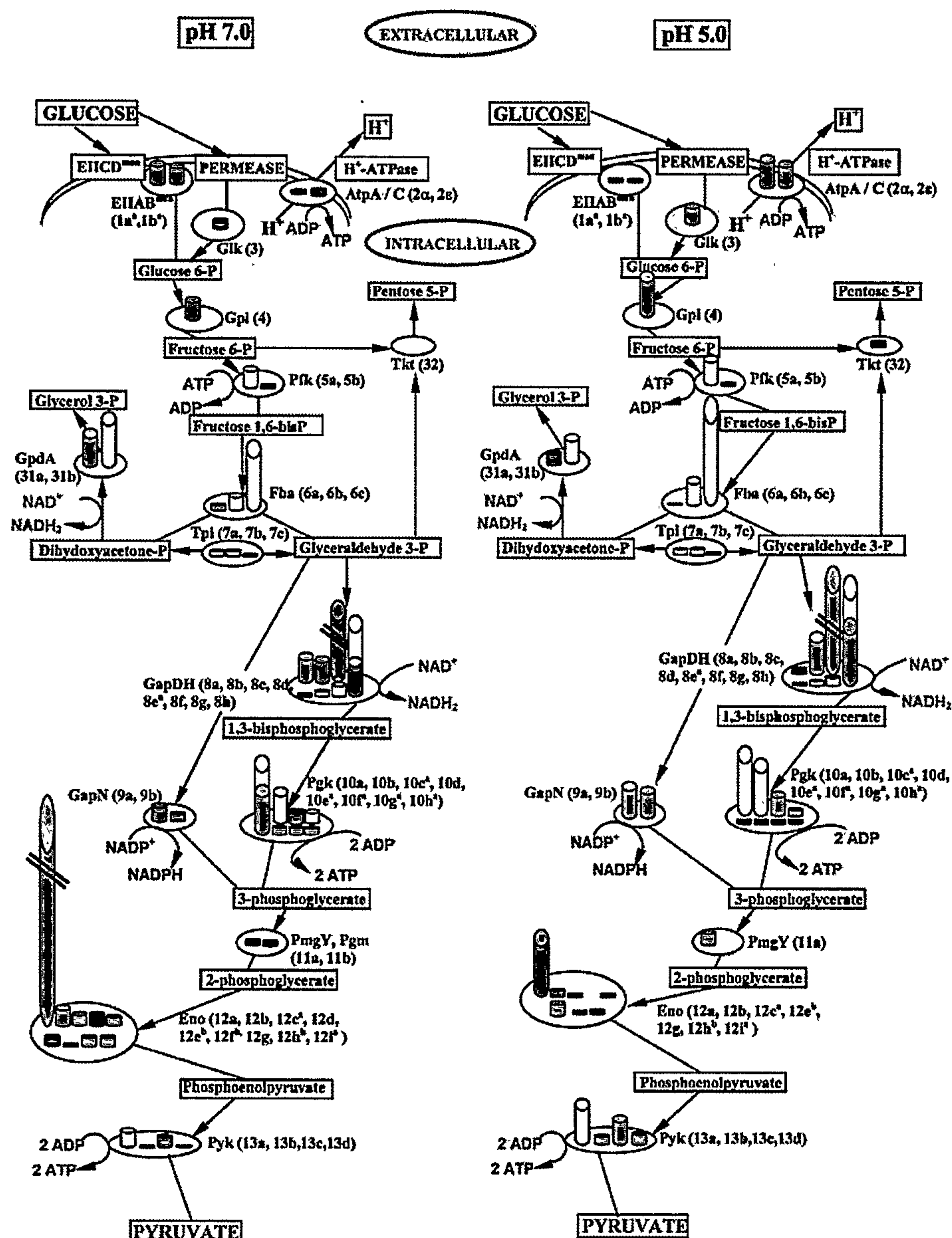


Fig. 1. Comparative expression, in cells grown at pH 7.0 or 5.0, of *S. mutans* proteins involved in glucose transport and glycolysis. Columns in ovals represent the relative mean DE value (arbitrary units) for each protein spot identified on 2-DGE gels. DE values greater than 100 000 are shown with a height break. At pH 5.0, protein spots were up-regulated (green), down-regulated (red), non-differentially expressed (<1.5-fold difference, grey), or uniquely expressed (blue), relative to pH 7.0. Membrane proteins not identified by proteome analyses are shown in light blue rectangles. Numbers in parentheses correspond to protein numbers in Table 1; superscripts identify protein spots as N-terminal (a) or C-terminal (b) fragments.

rate-limiting step in glycolysis when cells grown at pH 7.0 are resuspended at low pH *in vitro* (Iwami & Yamada, 1980). The observed increase in protein may therefore simply reflect a need to overcome this inhibition in cells growing at pH 5.0.

In studies of the aciduricity of *S. mutans*, the proton-translocating F_1F_0 -ATPase that extrudes H^+ has received

considerable attention, since streptococcal plasma membranes are proton-permeable (Bender *et al.*, 1986). A 7.5-fold increase in the α (AtpA) and 3.6-fold in the ϵ (AtpC) subunits of the F_1 -component of this complex was observed at pH 5.0 (Fig. 1, Table 1). This observation was consistent with previous data showing that levels of the H^+ -ATPase increase in *S. mutans* in response to acidification of the environment in order to maintain a transmembrane pH

gradient (ΔpH), with the interior of the cell more alkaline (Belli & Marquis, 1991; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992; Quivey *et al.*, 2001).

Glycolysis

Glucose entering the cell, by either the PEP-PTS or the permease/glucokinase pathway, enters the Embden–Meyerhof–Parnas glycolytic pathway as glucose 6-phosphate and is converted to pyruvate. Each of the enzymes in this pathway varied in the number of protein spots identified on 2-DGE gels (Fig. 1, Table 1).

Previous analysis of metabolic intermediates formed in the first half of the Embden–Meyerhof–Parnas pathway indicates that the level of glucose 6-phosphate is slightly elevated when *S. mutans* is grown in continuous culture at pH 5.5 and $D=0.15\text{ h}^{-1}$, while the levels of all other intermediates are essentially the same, up to and including dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Iwami *et al.*, 1992). On this basis, the doubling of glucose-6-phosphate isomerase (Gpi) and an overall 1.5-fold increase in the level of the three isoforms of fructose-1,6-bisphosphate aldolase (Fba), similar to that previously observed in batch culture (Wilkins *et al.*, 2002; Fig. 1, Table 1), possibly reflected a need to overcome the effect of cytoplasmic acidification on enzyme activity at pH 5.0. Some care should be exercised in such a simplified interpretation, however, as both fructose 6-phosphate and glyceraldehyde 3-phosphate may be required as substrates for transketolase (Tkt), in order to increase ribonucleic acid synthesis at pH 5.0 (see below).

Not only is energy in the form of ATP generated during glycolysis, but also precursors for anabolic reactions. For instance, *S. mutans* possesses a specific NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GapN) that bypasses the first ATP-generating step in glycolysis in order to generate the NADPH required for reductive biosynthetic reactions (Brown & Wittenberger, 1971a; Crow & Wittenberger, 1979; Boyd *et al.*, 1995; Fig. 1). This enzyme is essential, since *S. mutans* possesses neither the oxidative portion of the pentose phosphate cycle nor a transhydrogenase for the reduction of NADP by NADH (Brown & Wittenberger, 1971b). A 2.0- and 3.4-fold increase in the mean amount of the two isoforms of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase at pH 5.0, along with alterations in the levels of the isoforms of the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GapDH) and phosphoglycerate kinase (Pgm), highlighted the difficulty in determining the flux of carbon through the two branches of the pathway (Fig. 1, Table 1). However, since an overall 2.7-fold increase in NADP-dependent glyceraldehyde-3-phosphate dehydrogenase at pH 5.0 represented nearly twice the increase in the cumulative levels of each of the other two enzymes in the alternative oxidative branch of glycolysis, this suggested that NADP-dependent glyceraldehyde-3-phosphate dehydrogenase may indeed be of prime importance in determining this direction. This

would be in keeping with the high level of anabolic activity required to maintain the structural integrity of molecules such as proteins and DNA in a non-optimal acidic cytoplasm (Crow & Wittenberger, 1979; Quivey *et al.*, 2001; Len *et al.*, 2004). This notion was supported by the detection of a high level of transketolase (Tkt) solely on 2-DGE gels at pH 5.0. Transketolase forms a junction in the glycolytic pathway that redirects glyceraldehyde 3-phosphate and fructose 6-phosphate into the pentose phosphate pathway, and ultimately to nucleotide synthesis (Fig. 1, Table 1).

Three enzymes are involved in the final conversion of 3-phosphoglycerate to pyruvate. The first of these, phosphoglyceromutase, is represented by six alleles in the *S. mutans* genome (Ajdić *et al.*, 2002). Of the two protein products detected, one (PmgY) was up-regulated 2.7-fold at pH 5.0, while the other (Pgm) was absent at this pH (Fig. 1, Table 1). The four isoforms of the final glycolytic enzyme, pyruvate kinase (Pyk), were also up-regulated at pH 5.0 by 2.4-, 7.1-, 2.3- and 7.8-fold, respectively. Pyruvate kinase is believed to be the key rate-limiting step in the regulation of glycolysis in *S. mutans*, since it is activated by glucose 6-phosphate but inhibited by inorganic phosphate (Yamada & Carlsson, 1975b; Abbe & Yamada, 1982; Iwami & Yamada, 1980). However, the 93 % reduction of the dominant form of the preceding enzyme in the Embden–Meyerhof–Parnas glycolytic pathway, enolase (Eno), along with reductions in the other minor high- M_r and truncated forms, would be expected to have a significant effect on carbon flow at pH 5.0, especially as the pH optimum for enolase activity is 7.5 (Bunick & Kashet, 1981).

As *S. mutans* growing in continuous culture is devoid of measurable glucose PEP-PTS activity at pH 5.0 (Vadeboncoeur *et al.*, 1991), phosphoenolpyruvate is not required to initiate the phosphorylation cascade for this glucose uptake system. The levels of the final three enzymes in glycolysis at pH 5.0 were consistent with a reduced pool of phosphoenolpyruvate, since the reduced levels of enolase, in conjunction with an overall 2.8-fold increase in the four isoforms of pyruvate kinase, would be expected to convert 2-phosphoglycerate to pyruvate without any build-up of phosphoenolpyruvate. This idea is supported by the measurement of the four intermediates, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate, in *S. mutans* grown at pH 5.5 and $D=0.15\text{ h}^{-1}$, where the levels of 3-phosphoglycerate and phosphoenolpyruvate decrease, that of 2-phosphoglycerate remains static and the amount of pyruvate increases (Iwami *et al.*, 1992). This is further supported by another study, where a fall in intracellular pH was shown to significantly increase the pyruvate:phosphoenolpyruvate ratio (Iwami & Yamada, 1980). Phosphoenolpyruvate is also an inhibitor of L-lactate dehydrogenase (Yamada & Carlsson, 1975b; Yamada, 1987), so low levels of this metabolite would allow a higher concentration of lactate to accumulate at pH 5.0, as was indeed the case (Table 2; see below).

Table 2. Effect of growth pH on the yield and end-products of metabolism in *S. mutans*

Figures represent the mean of at least three determinations. Y_{ATP} was determined according to the assumptions of Harty & Handley (1988).

Growth pH	Y_{Glc} (g dry wt mol ⁻¹)	Y_{ATP} (g dry wt mol ⁻¹)	IPS [μ g (mg dry wt) ⁻¹]	End product [mmol (mol glucose utilized) ⁻¹]				
				D-Lactate	L-Lactate	Formate	Acetate	Ethanol
7.0	28.0	16.9	11.2	64	6	1156	640	368
5.0	15.1	3.9	10.9	68	760	1328	732	1688

Regeneration of NAD

In *S. mutans*, pyruvate can be converted into a variety of acidic end products (Fig. 2). In an aerobic environment, *S. mutans* utilizes pyruvate dehydrogenase to convert pyruvate to acetyl-CoA (Carlsson *et al.*, 1985). Despite the fact that this enzyme should be inactive under the anaerobic conditions of growth utilized in this study, two of the three components of the multi-enzyme pyruvate dehydrogenase complex, the acetoin dehydrogenase E1 component, (AcoB), and a C-terminal fragment of dihydrolipoamide S-acetyltransferase (AdhC), were identified on 2-DGE gels (Fig. 2, Table 1).

Under anaerobic conditions, and in the presence of excess glucose, L-lactic acid is formed by L-lactate dehydrogenase (Ldh). In reducing pyruvate to L-lactate, NADH is oxidized to NAD and the oxidation–reduction balance of the cell is preserved. However, under anaerobic conditions, when glucose is limiting, the bacterium relies on the pyruvate formate-lyase pathway, which has two branches, leading either to the formation of formate and acetate or to the formation of ethanol (Carlsson & Griffith, 1974; Yamada & Carlsson, 1975b; Yamada, 1987). The ethanol branch involves two steps, in which 2 NADH are oxidized to 2 NAD. In *S. mutans*, these two steps, in which acetyl-CoA is first converted to acetaldehyde and then to ethanol, are catalysed by the bifunctional enzyme, alcohol-acetaldehyde dehydrogenase (AdhE). In the acetate branch of the pathway, an extra ATP is generated, as acetyl-CoA is first converted to acetyl phosphate and then to acetate, with the concomitant conversion of ADP to ATP. By appropriate use of the acetate and ethanol branches of the pyruvate-formate lyase pathway, *S. mutans* can readily adjust NADH oxidation to actual need. Without further anabolic diversion of the final products, the amount of formate produced by glycolysis is equivalent to the total amount of acetate and ethanol.

Proteome analysis showed that the two isoforms of L-lactate dehydrogenase detected on 2-DGE gels were up-regulated at pH 5.0 by 2.5- and 1.7-fold, compared with the levels at pH 7.0. These values are similar to reports of a total 1.7-fold increase observed on 2-DGE gels for the three isoforms of L-lactate dehydrogenase in *S. mutans* and the five isoforms in *S. oralis* when grown without pH control in batch culture (Wilkins *et al.*, 2001, 2002). The increase in L-lactate dehydrogenase isoforms observed in our study is unlikely to account for the 127-fold increase in the amount of L-lactic

acid produced under acidic conditions (Table 2), despite the fact that L-lactate dehydrogenase has a broad optimum for catalytic activity in the acidic pH range 5.0–6.2, similar to the pH range 6.0–6.5 of the cytoplasm of cells growing at pH 5.0 (Dashper & Reynolds, 1990; Hamilton, 1990; Iwami *et al.*, 1992). The more likely explanation is that the enzyme is regulated by glycolytic intermediates at the lower growth pH (Yamada & Carlsson, 1975b; Takahashi *et al.*, 1982; Yamada, 1987).

While no change in the level of expression of pyruvate formate-lyase was observed in the current study, since the enzyme was not detected on 2-DGE gels (Fig. 2, Table 1), the six isoforms of alcohol-acetaldehyde dehydrogenase were, on average, down-regulated 7.0-fold at pH 5.0 while the numerous C-terminal truncated forms were either down-regulated or missing at this pH. Acetate kinase (AckA), in the acetate branch, was also down-regulated by a factor of 2.1. In contrast, the level of phosphotransacetylase increased 1.7-fold at pH 5.0 (Pta; Fig. 2, Table 1). Despite the general overall reduction in protein expression observed on 2-DGE gels in these two branches of the pyruvate formate-lyase pathway, increased amounts of formate, acetate and ethanol were produced at pH 5.0, with the level of ethanol being 4.6-fold greater than at pH 7.0 (Table 2). This observation may be explained mainly by the increased glycolytic rate (Hamilton, 1984) needed to produce ATP for H⁺ extrusion at pH 5.0 (Bender *et al.*, 1986; Belli & Marquis, 1991; Hamilton & Buckley, 1991; Miyagi *et al.*, 1994; Smith *et al.*, 1996). The fact that there was a futile use of carbon at pH 5.0 was reflected in the measured reduction in both ATP yield, (Y_{ATP} ; Harty & Handley, 1988) from 16.9 to 3.9 g (dry weight) cells per mol ATP, and the cell yield (Y_{Glc}), from 28.0 to 15.1 g (dry weight) cells per mol glucose, when the growth pH was reduced from 7.0 to 5.0 (Table 2). While clearly not preventing the production of alternative acids, the question remains whether the reduction in enzyme levels in the ethanol and acetate branches of the pyruvate formate-lyase pathway becomes limiting in cells growing at pH 5.0, and by so doing enhances the flow of pyruvate through the L-lactate dehydrogenase pathway.

Formation of branched-chain amino acids

A decrease in the formate:(acetate + ethanol) ratio, from the theoretical maximum glycolytic value of 1.0 to a value of 0.56, was observed at pH 5.0 (Table 2). Since the

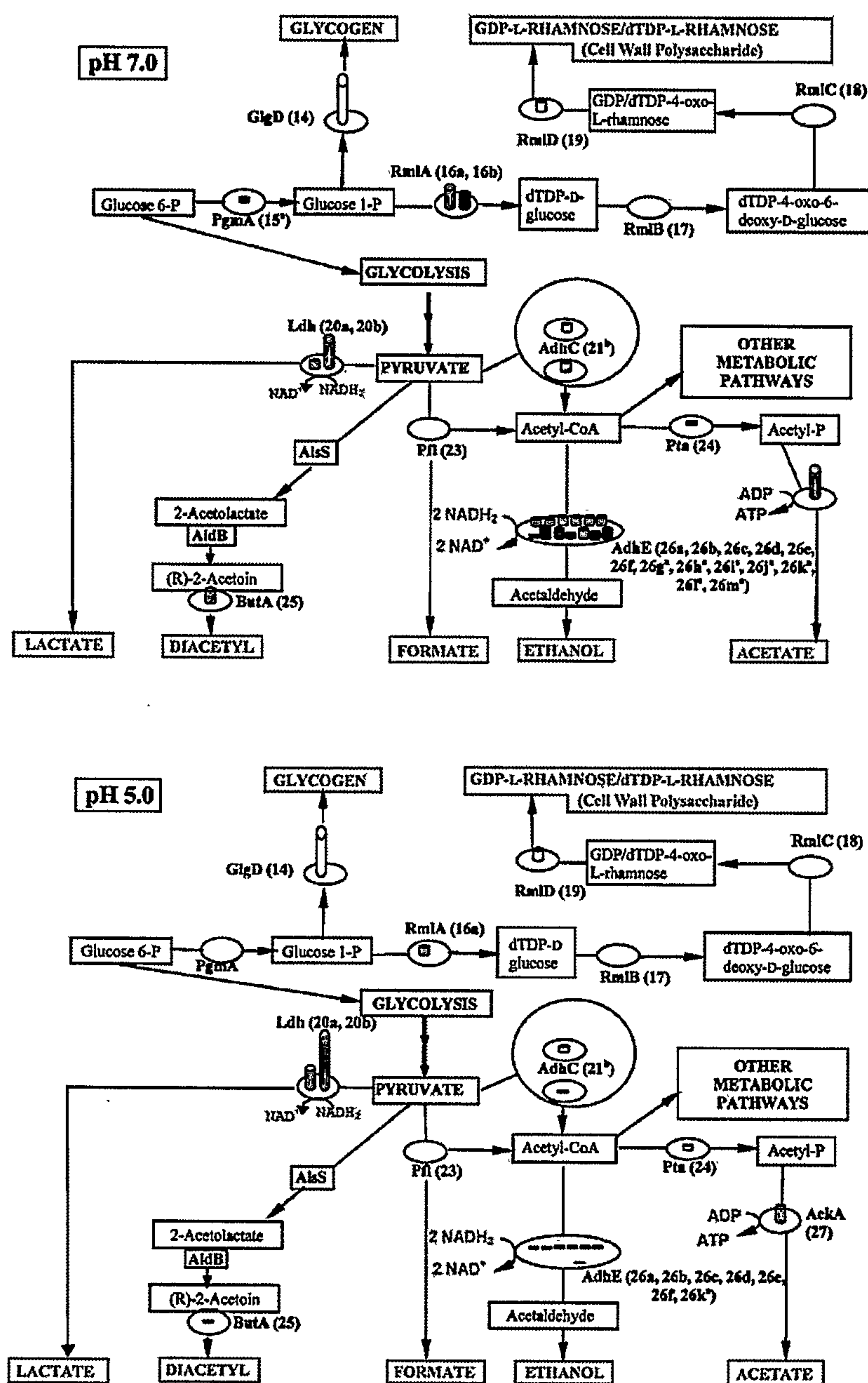


Fig. 2. Comparative expression, in cells grown at pH 7.0 or 5.0, of *S. mutans* proteins involved in NADH oxidation and the alternative use of glucose 6-phosphate. Columns in ovals represent the relative mean DE value (arbitrary units) for each protein spot identified on 2-DGE gels. At pH 5.0, protein spots were up-regulated (green), down-regulated (red), non-differentially expressed (<1.5-fold difference, grey), or uniquely expressed (blue), relative to pH 7.0. Membrane proteins not identified by proteome analyses are shown in light blue rectangles, while those in tan ovals were identified previously, (Len *et al.*, 2003). Numbers in parentheses correspond to protein numbers in Table 1; superscripts identify protein spots as N-terminal (a) or C-terminal (b) fragments.

concentration of acetate was similar at both pHs, the relative reduction in formate at pH 5.0 suggested that *S. mutans* was further metabolizing this acid. The alternative scenario, of an increase in ethanol production by another catabolic

process, could be ruled out, since ethanol can only be formed via the pyruvate formate-lyase pathway. Comparative proteome analysis suggested that the fate of some of the formate at pH 5.0 lay with the branched-chain amino acid

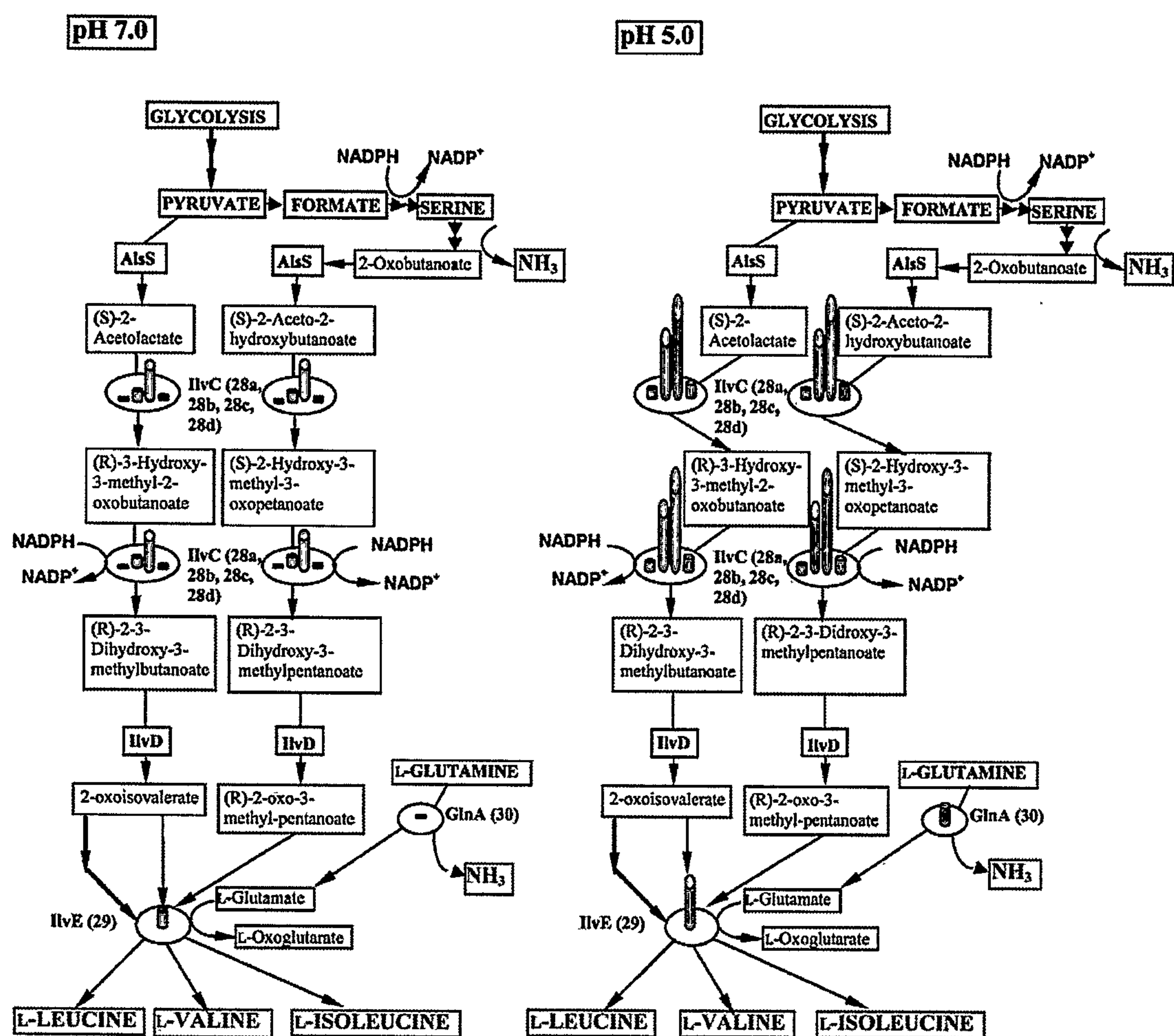


Fig. 3. Comparative expression, in cells grown at pH 7.0 or 5.0, of *S. mutans* proteins involved in branched-chain amino acid biosynthesis. Columns in ovals represent the relative mean DE value (arbitrary units) for each protein spot identified on 2-DGE gels. Protein spots were up-regulated at pH 5.0 (green), and down-regulated at pH 7.0 (red). Proteins not identified by proteome analyses are shown in light blue rectangles. Numbers in parentheses correspond to protein numbers in Table 1.

biosynthesis pathway, since an increase was observed in the enzymes associated with this amino acid pathway alone at pH 5.0 (Fig. 3 and Table 1). The connection arises, since isoleucine biosynthesis requires 2-oxobutanoate, which is obtained by deamidation of serine by threonine ammonium lyase (IlvA, EC 4.3.1.19). Serine is formed from formate by way of the one-carbon donor cycle. The biosynthesis of both leucine and valine, on the other hand, relies directly on pyruvate (Fig. 3). Ultimately, however, branched-chain amino acid biosynthesis requires both pyruvate and NADPH for the synthesis of leucine, isoleucine and valine. *De novo* synthesis of these amino acids would reduce acid production in two ways, directly by removing reducing equivalents in the form of pyruvate and 2-oxobutanoate, and indirectly by the consumption of NADPH (Fig. 3). Furthermore, reducing the level of formate also reduces the concentration of H^+ , since formic acid is a stronger acid (pK_a 3.75) than either lactic acid (pK_a 3.86) or acetic acid (pK_a 4.75).

At pH 5.0, the four isoforms of ketol-acid reductoisomerase (IlvC) were up-regulated 3.9-, 5.7-, 2.6- and 3.5-fold, while branched-chain amino acid aminotransferase (IlvE) and glutamine synthetase (GlnA) were up-regulated 2.7- and 8.7-fold, respectively (Fig. 3, Table 1). The product of branched-chain amino acid aminotransferase, 2-oxoglutaric acid (α -ketoglutaric acid), is also a weaker acid than glutamate, with pK_a values of 2.8 and 5.0, compared with pK_a values of 2.2 and 4.2 for the carboxyl groups of glutamic acid. Formation of branched-chain amino acids would have an added advantage, since the biosynthesis of all three amino acids involves the formation of NH_3 by glutamine synthetase. This NH_3 , along with that produced from serine during the biosynthesis of isoleucine, would provide an alternative mechanism for buffering an acidic cytoplasm in *S. mutans* at a growth pH of 5.0, since the NH_3 would react with H^+ to form NH_4^+ (Fig. 3). Interestingly, pyruvate kinase activity has an obligatory requirement for either K^+

or NH_4^+ , and a high concentration of NH_4^+ does not seem to be detrimental to the growth of oral streptococci (Abbe & Yamada, 1982; Yamada, 1987; Pitty & Jacques, 1989).

Support for a role for branched-chain amino acid biosynthesis in the survival of streptococci at low pH comes from two recent observations. In *Streptococcus thermophilus*, the branched-chain amino acid biosynthesis pathway has been found to be necessary but not sufficient to ensure optimal growth, and a role has been postulated for the pathway in maintaining internal pH (Garault *et al.*, 2000). Differential-display RT-PCR has also demonstrated that branched-chain amino acid aminotransferase is up-regulated by acidic conditions in *S. mutans* and that inactivation of *ilvC* leads to retarded growth at pH 5.5 (Chia *et al.*, 2001). Further support for a role for branched-chain amino acid synthesis in maintaining internal pH comes from the fact that the 2-acetolactate that is synthesized from pyruvate by acetolactate synthase (AlsS, EC 2.2.1.6) occurs at the junction of the leucine/valine and acetoin biosynthesis pathways. The acetoin biosynthesis pathway leads to the formation of diacetyl (Fig. 2). While only the last enzyme in this pathway, acetoin dehydrogenase (ButA), was identified on 2-DGE gels, the amount of the enzyme at pH 5.0 was only 3% of that at pH 7.0 (Table 1), suggesting that most of the 2-acetolactate had been directed to the leucine/valine biosynthesis pathway.

Although the proteome data, in concert with the acid and ethanol end-product analyses, were consistent with the formation of branched-chain amino acids, the concentration of these amino acids in the continuous-culture medium was only 2% higher at pH 5.0 than at pH 7.0 (data not shown). Unlike other amino acid transport systems in *S. mutans*, branched-chain amino acid uptake has been well studied and has been shown to be driven by the proton-motive force, which results in high intracellular branched-chain amino acid levels (Dashper & Reynolds, 1990). Loss of these amino acids from the cell is due to passive efflux through the plasma membrane (Dashper & Reynolds, 1990). As the residual extracellular concentration of each of the branched-chain amino acids is approximately 0.3 mM at steady state in continuous culture, their cellular concentration would be as high as 10 mM (Dashper & Reynolds, 1990). As a consequence, an increase in the cellular biosynthesis of these amino acids at pH 5.0 would not necessarily have been reflected in an increase in extracellular levels. Also worthy of note is that analysis of codon usage shows that 24.4% of the amino acids in the proteome of *S. mutans* are branched-chain amino acids, implying that there is a high biosynthetic requirement for these amino acids in protein synthesis.

Alterations in other biosynthetic pathways

Changes in the level of expression of individual proteins, representing incomplete metabolic pathways, were also identified by 2-DGE. The first of these was associated with the utilization of glucose 1-phosphate, following its

conversion from glucose 6-phosphate by phosphoglucosyltransferase (PgmA). A C-terminally truncated form of this enzyme was detected at pH 7.0 only (Fig. 2, Table 1). Glucose 1-phosphate, which is the initial substrate for intracellular polysaccharide synthesis, was synthesized to only a limited extent at either pH, consistent with glucose being limiting at $D=0.1 \text{ h}^{-1}$ (Hamilton, 1984; Table 2). Glucose 1-phosphate is also the initial substrate for four enzymes associated with the biosynthesis of rhamnose, a sugar component of *S. mutans* cell-wall polysaccharide (Schleifer & Kilpper-Bälz, 1987). While the first of these enzymes, glucose-1-phosphate thymidyltransferase (RmlA), existed as two isoforms at pH 7.0, only one was detected at pH 5.0, at a 2.4-fold lower level (Fig. 2, Table 1). The other three enzymes were not observed to be differentially expressed at the lower pH (Fig. 2, Table 1).

The second pathway was represented by only one protein: two isoforms of the NAD-dependent glycerol-3-phosphate dehydrogenase (GpdA), which forms the last step in glycerolipid degradation, directing carbon into the glycolytic pathway (Fig. 1). The levels of the two isoforms were reduced by factors of 2.7- and 1.9, respectively, at pH 5.0 (Table 1).

The third pathway was also represented by only one enzyme, lactoylglutathione lyase (GloA). This pathway constitutes a bypass in glycolysis, which converts the reactive dicarbonyl compound methylglyoxal into D-lactate. Methylglyoxal is toxic, as it can interact with and modify both nucleic acids and proteins (Thornalley, 1996). Methylglyoxal is formed from dihydroxyacetone and glyceraldehyde 3-phosphate, with or without catalysis by triose-phosphate isomerase. While the amount of D-lactate produced by *S. mutans* was constant, irrespective of the growth pH (Table 2), the level of lactoylglutathione lyase was increased 2.6-fold at pH 5.0 (Table 1). This level of up-regulation was far less than the 51-fold increase reported for *S. mutans* grown in batch culture without pH control (Wilkins *et al.*, 2002).

Concluding remarks

Changes in the protein phenotype of *S. mutans*, leading to acid tolerance at pH 5.0, have been made possible by using a standardized, continuous-culture technique that enables a snapshot of the cellular proteome to be obtained during steady state. This differs from the situation in non-regulated batch cultures, where environmental changes are ongoing throughout growth and stationary phase. Continuous culture, in combination with enhanced 2-DGE proteome analysis with both mid-range and narrow-range IPG strips, has thus enabled us to measure the changes that take place, following a transition to an acidic environment, in the expression of metabolic proteins by *S. mutans*. While the cumulative changes in protein levels clearly impact on the flux of carbon through a given metabolic pathway, such changes cannot be viewed in isolation. Allosteric regulation, and also the stability and relative activity of a given enzyme at a given cytoplasmic pH, are important criteria in

establishing the ultimate metabolic flux of carbon through a pathway (Yamada & Carlsson, 1975a; Yamada, 1987). Nevertheless, this study has highlighted the extent to which changes occur in the levels of enzymes involved in central metabolism.

Studies of changes in the levels of protein expression, along with measurement of the final by-products of carbon utilization, Y_{Glc} and Y_{ATP} , have confirmed previous findings that streptococci tolerate growth at pH 5.0 by increasing their ability to extrude H^+ , while modulating the production of acid fermentation by-products to reduce the level of H^+ production (Hamilton, 1984; Iwami *et al.*, 1992; Miyagi *et al.*, 1994). The results further suggest that *S. mutans* utilizes branched-chain amino acid biosynthesis to lower the amount of reducing equivalents in the form of NAD(P)H that would otherwise be converted to acidic by-products, and by so doing helps buffer the cytoplasm by producing NH_3 . The exact role of branched-chain amino acid biosynthesis in the acid resistance of *S. mutans*, and the validity or otherwise of this hypothesis, is currently the subject of further investigation in our laboratory. The observed alterations in the levels of expression of proteins, however, clearly reflect the complexity required to maintain a phenotype commensurate with the survival of *S. mutans* in an acidic environment. In this regard, the nature of the multiple protein forms (particularly isoforms) remains to be elucidated, as do the mechanism(s) by which such an integrated change in the level of proteins is coordinated.

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Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance

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Streptococcus mutans is an important pathogen in the initiation of dental caries as the bacterium remains metabolically active when the environment becomes acidic. The mechanisms underlying this ability to survive and proliferate at low pH remain an area of intense investigation. Differential two-dimensional electrophoretic proteome analysis of *S. mutans* grown at steady state in continuous culture at pH 7.0 or pH 5.0 enabled the resolution of 199 cellular and extracellular protein spots with altered levels of expression. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry identified 167 of these protein spots. Sixty-one were associated with stress-responsive pathways involved in DNA replication, transcription, translation, protein folding and proteolysis. The 61 protein spots represented isoforms or cleavage products of 30 different proteins, of which 25 were either upregulated or uniquely expressed during acid-tolerant growth at pH 5.0. Among the unique and upregulated proteins were five that have not been previously identified as being associated with acid tolerance in *S. mutans* and/or which have not been studied in any detail in oral streptococci. These were the single-stranded DNA-binding protein, Ssb, the transcription elongation factor, GreA, the RNA exonuclease, polyribonucleotide nucleotidyltransferase (PnpA), and two proteinases, the ATP-binding subunit, ClpL, of the Clp family of proteinases and a proteinase encoded by the *pep* gene family with properties similar to the dipeptidase, PepD, of *Lactobacillus helveticus*. The identification of these and other differentially expressed proteins associated with an acid-tolerant-growth phenotype provides new information on targets for mutagenic studies that will allow the future assessment of their physiological significance in the survival and proliferation of *S. mutans* in low pH environments.

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INTRODUCTION

Streptococcus mutans is now well recognized as being associated with the initiation of dental caries, since its acid fermentation by-products can result in the demineralization of tooth enamel (Hamada & Slade, 1980; Harper & Loesche, 1984; Loesche, 1986; van Houte, 1994; van Ruyven *et al.*, 2000). A key to the survival of *S. mutans* at low pH is its ability to maintain a transmembrane pH gradient (Δ pH), with the interior of the cell more alkaline. This is achieved by upregulation of a proton-translocating F_1F_0 -ATPase that extrudes H^+ as the external environment becomes more acidic. This results in an increased use of ATP for H^+ extrusion and a consequent reduction in cell yield (Belli & Marquis, 1991; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992; Quivey *et al.*, 2001). A series of recent

physiological, mutagenic and proteome studies (Quivey *et al.*, 1995; Gutierrez *et al.*, 1996, 1999; Jayaraman *et al.*, 1997; Hamilton & Svensäter, 1998; Hahn *et al.*, 1999; Hanna *et al.*, 2001; Kremer *et al.*, 2001; Lemos *et al.*, 2001; Li *et al.*, 2002; Wilkins *et al.*, 2002; Len *et al.*, 2004), however, indicates that *S. mutans* regulates its phenotype in a far more complex fashion than simply increasing its ability to extrude H^+ in response to acid stress. For instance, our recent proteome analysis detected changes in metabolic pathways following acid-tolerant growth. Analysis of the data gave rise to the hypothesis that *S. mutans* redirects carbon from acidic fermentation by-products to more alkaline catabolites. These changes appear to occur in order to minimize the detrimental effects that result from the uncoupling of carbon flux from catabolism, as a consequence of the use of ATP for H^+ extrusion (Len *et al.*, 2004). What remains to be elucidated is the breadth of the stress response in *S. mutans* that allows it to survive and proliferate at low pH.

Here we report the phenotypic changes, previously associated with the maintenance of bacterial viability

Abbreviations: 2-DGE, two-dimensional gel electrophoresis; ASB-14, amidosulfobetaine-14; *D*, dilution rate; DE, differential expression (values); IPG, immobilized pH gradient; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMM, peptide mass mapping.

under a variety of imposed environmental stresses, that were observed when *S. mutans* was grown at steady state in continuous culture at low pH. Proteins required for the maintenance of DNA integrity, transcriptional fidelity, translational efficiency, and protein folding were uniquely identified during acid-tolerant growth at pH 5.0, or were present at higher levels than those in *S. mutans* grown at pH 7.0. The mode of action of these proteins is discussed in relation to current knowledge of their roles in responding to stress, particularly in Gram-positive bacteria associated with acidic environments.

METHODS

Growth conditions. Triplicate continuous cultures of *Streptococcus mutans* LT11 (Tao *et al.*, 1993) were grown under anaerobic conditions at a dilution rate (D) of $0.100 \pm 0.001 \text{ h}^{-1}$, at either pH 7.0 ± 0.1 or pH 5.0 ± 0.1 , with glucose limitation as previously described (Jacques *et al.*, 1979). DMM medium, devoid of mucin, was used, but modified to include adenine, guanine and uracil at $20 \mu\text{g ml}^{-1}$, and both KH_2PO_4 and K_2HPO_4 at 15 mM (Sissons *et al.*, 1991).

Preparation of cellular and extracellular proteins. When steady state had been achieved, the bacterial contents of the culture vessel were harvested, washed and lyophilized before aliquots of 10 mg dry wt of cells were treated with mutanolysin (Len *et al.*, 2003). Proteins that were to be separated on acidic immobilized pH gradient (IPG) strips (pH 4.0–6.7) were extracted as previously described (Len *et al.*, 2003), except that 1% (w/v) amidosulfo betaine-14 (ASB-14) and 65 mM DTT were added to produce a modified solubilizing solution for two-dimensional electrophoresis (2-DGE). While the addition of these reagents increased the total number of protein spots that could be readily discerned on 2-DGE gels, their inclusion selectively inhibited the extraction or subsequent separation of a small number of weakly expressed proteins that had been previously visualized and/or identified on 2-DGE gels (Len *et al.*, 2003).

Proteins that were to be separated on basic IPG strips (pH 6–11) were obtained from the mutanolysin-treated cells by a two-fraction solubilization procedure. Following centrifugation of the cell lysate (12 000 g, 4 °C, 10 min), the cell pellet was stored at –20 °C, and the proteins in the supernatant precipitated overnight at –20 °C with 15% (w/v) trichloroacetic acid. After centrifugation and two washes in methanol (12 000 g, 4 °C, 10 min), the precipitated proteins were solubilized in 300 μl of a 1:1 mixture of modified solubilization solution (without ASB-14) and Cellular and Organelle Membrane Solubilizing Reagent (Sigma-Aldrich) containing 1% (v/v) Triton X-100 and 2 mM tributylphosphine. The frozen cell pellet was then thawed and resuspended by sonication (Branson Ultrasonics; 50 W, $10 \times 10 \text{ s}$, 20–22 °C, with cooling on ice between each burst) in 700 μl of the same solubilization solution, before 150 U of exonuclease III was added and the suspension incubated at room temperature (20–22 °C) for 15 min to degrade any DNA. The two cellular fractions were then combined and centrifuged at room temperature (12 000 g, 20–22 °C, 10 min). Prior to IEF, 100 μl 500 mM iodoacetamide was added, and the mixture incubated at room temperature (20–22 °C) for 2 h.

2-DGE and mass spectroscopic analyses of proteins. Both 2-DGE and mass spectroscopic analyses, using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, were performed using a PerSeptive Biosystems Voyager DE-STR mass spectrometer, with trypsin autolysis peptide masses of 842.5 and 2211.1 Da as internal standards, as previously described (Len *et al.*, 2003, 2004). All mass spectra were obtained in reflectron-delayed

extraction mode. The density/volume, or differential expression (DE) value (arbitrary units), of SyproRuby-stained protein spots of 2-DGE gels was determined using the software package z3 (Compugen). The main source of error associated with this form of quantification is the reproducibility of the 2-DGE displays themselves, as biological variation is minimized when a chemostat is used to culture bacteria. That the 2-DGE displays were the main source of error was confirmed by comparing the DE values of 25 randomly chosen protein spots selected from 2-DGE displays separated on broad-range IPG strips over the pI range 4.0–7.0. Equivalent protein spots from triplicate samples from each of three repeat continuous cultures were analysed. The data confirmed that the main source of error in determining DE values was 2-DGE. As a consequence, triplicate experimental samples from cells grown at each pH were used for all 2-DGE analyses, and the increase in the level of expression of a protein spot was based on the difference in the mean DE values.

Protein identification. Peptide mass mapping (PMM) analyses of proteins were undertaken as previously described, making use of the six contigs of the *S. mutans* UA159 genome downloaded on October 6, 2001 that were translated in all six reading frames (Len *et al.*, 2003). All translated ORFs that matched PMM data were then used to query the annotated *S. mutans* genome at the Oral Pathogen Sequence Databases (<http://www.stdgen.lanl.gov/oragen>; Ajdić *et al.*, 2002), using the local BLAST search facility to determine the gene identification number. The original six contigs were used in this manner as some genes identified in these contigs were not present in the final annotated version.

Mass spectroscopic parameters for protein identification included a mass tolerance of 150 p.p.m. and a maximum of one missed cleavage per peptide while taking into consideration methionine sulfoxide and cysteine acrylamide modifications. Matches were defined on the basis of the number of matching peptide masses and the total percentage sequence covered by the peptides. As a general rule, a minimum total sequence coverage of 25% was taken to match a given translated ORF of a high- M_r protein with confidence, though coverage as high as 80% was observed with many low- M_r proteins. All translated ORFs that matched PMM data were then used to query the annotated *S. mutans* genome at the Oral Pathogen Sequence Databases (<http://www.stdgen.lanl.gov/oragen>), using the local BLAST search facility to determine the gene identification number. All gene names used are those associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site. Theoretical M_r and pI were determined using MassLynx software version 3.4 (Micromass).

Protein isoforms. The term 'isoform' is used in the text to describe a protein that exists in multiple charged forms on 2-DGE gels, where the mean observed M_r for each form calculated from the second (SDS-PAGE) dimension deviates by up to 5% and where there is no evidence from peptide mass mapping of any form of truncation or degradation (Len *et al.*, 2004).

RESULTS AND DISCUSSION

The increased expression of the proton-translocating F_1F_0 -ATPase that extrudes H^+ from *Streptococcus mutans* (Belli & Marquis, 1991; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992; Quivey *et al.*, 2001) is a key to the survival of the bacterium in an acidic environment. This was reflected in the previously reported increase in the α - (AtpA) and γ - (AtpC) subunits of the F_1 component of the ATPase when the bacterium was grown at pH 5.0 (Fig. 1, Table 1; Quivey *et al.*, 2001; Len *et al.*, 2004). A combination of steady-state continuous-culture technology and narrow-range IPG strips, however, resulted in the resolution of an additional 197

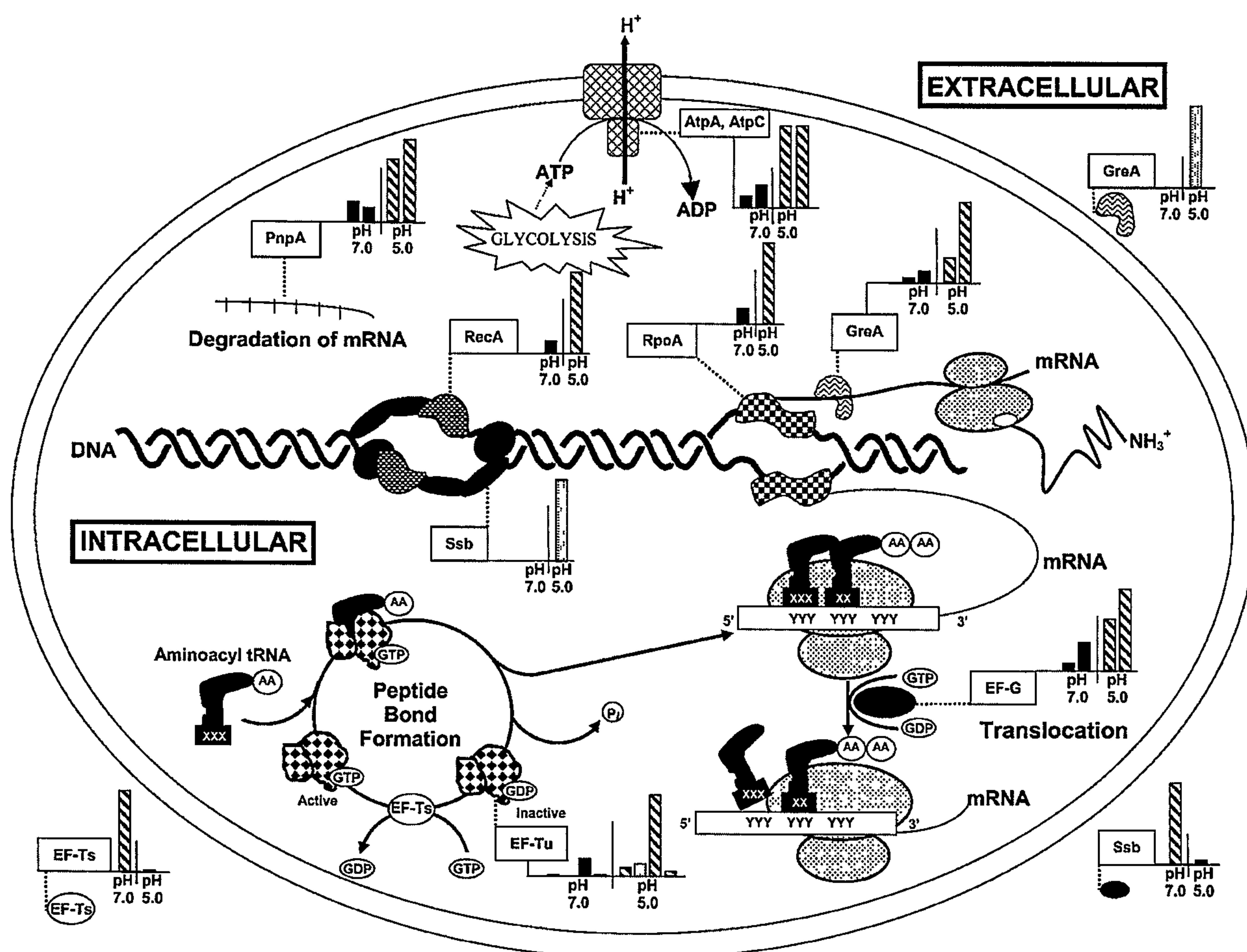


Fig. 1. Differentially expressed *S. mutans* proteins involved in replication, transcription and translation from cells grown at pH 7.0 or at pH 5.0. The columns represent the percentage mean DE values for each charged isoform identified on 2-DGE gels, relative to the most highly expressed isoform. Protein spots were either upregulated (cross-hatching), down-regulated (black), or uniquely expressed (dots), relative to the alternative pH. Truncated forms of proteins are not shown, except for those observed in the extracellular milieu.

differentially expressed protein spots on SyproRuby-stained 2-DGE gels, following acid-tolerant growth of *S. mutans* at pH 5.0. Of these, 167 (including all 44 extracellular protein spots) were identified by MALDI-TOF analysis, and 106 found to be associated with metabolism: glycolysis, alternative acid production and branched-chain amino acid synthesis, in particular (Len *et al.*, 2004). The remaining 61 protein spots were associated with regulatory and/or stress-responsive pathways. These proteins included those involved in DNA replication, transcription, translation, protein folding and proteolysis and are discussed below in light of current knowledge of the possible roles they play in Gram-positive bacteria, particularly oral streptococci.

DNA replication and chromosome integrity

One of the consequences of intracellular acidification is the loss of purines and pyrimidines from DNA, since

deoxyribonucleotides are acid labile (Lindahl & Nyberg, 1972). Unrepaired non-instructive DNA damage blocks DNA replication and can be lethal. Aborted replication, however, exposes single-stranded DNA at replication forks and results in the binding of the recombinase RecA. The DNA-bound RecA protein undergoes a conformational transition to its active form, RecA*. In both *Escherichia coli* and *Streptococcus pneumoniae*, RecA* induces an SOS response that can lead to mutation of the DNA or acquisition of pre-evolved functions by horizontal gene transfer (Taddei *et al.*, 1997; Horst *et al.*, 1999; Steffen & Bryant, 2000; Volkert & Landini, 2001; Bjedov *et al.*, 2003; Katz & Bryant, 2003).

In *S. mutans*, RecA was found to be upregulated 6.8-fold when grown at pH 5.0 (Fig. 1, Table 1). The physiological effects of low pH on a RecA-deficient strain of *S. mutans* have previously indicated that RecA is required for survival

Table 1. Differentially expressed cellular stress-related proteins from *S. mutans* grown at pH 7.0 or pH 5.0

Numbers in the Gene ID column are associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site (<http://www.stdgen.lanl.gov/oragen>).

Gene ID	Protein, EC no. (where applicable) and gene	2-DGE coordinates (pI/M _r)		DE value‡	
		Theoretical	Observed‡	pH 7.0	pH 5.0
Energy metabolism					
ATPase/proton motive force					
SMu1391	ATP synthase, alpha chain (EC 3.6.3.14) (<i>atpA</i>)	5.0/49 350	4.87 ± 0.02/53 310 ± 180	1 076 ± 417	8 105 ± 229
SMu1388	ATP synthase, epsilon chain (EC 3.6.3.14) (<i>atpC</i>)	6.50/15 882	6.50/15 880§	1 817 ± 567	6 468 ± 549
DNA replication					
DNA replication, restriction, modification, recombination and repair					
SMu1892	Recombinase A (<i>recA</i>)	5.0/36 987	5.40 ± 0.00/48 280 ± 480	300 ± 157	2 040 ± 661
SMu0268	DNA-directed DNA polymerase I (EC 2.7.7.7) (<i>polA</i>)*	5.3/99 455	4.70 ± 0.00/43 110 ± 370	764 ± 334	0.0
SMu1693	Single-stranded DNA-binding protein (<i>ssb</i>)	5.0/18 332	4.93 ± 0.03/17 930 ± 40	0.0	3 773 ± 183
Transcription					
RNA synthesis and modification					
SMu1817	DNA-directed RNA polymerase, alpha subunit (EC 2.7.7.6) (<i>rpoA</i>)	3.8/34 571	4.80 ± 0.00/41 340 ± 130	1 753 ± 472	9 428 ± 549
SMu1574	Transcription elongation factor (<i>greA</i>)	4.8/17 586	4.80 ± 0.00/15 870 ± 30	2 631 ± 495	19 600 ± 692
SMu1574	Transcription elongation factor (<i>greA</i>)	4.8/17 586	4.80 ± 0.00/17 590 ± 0.0	1 137 ± 131	5 775 ± 395
Degradation of RNA					
SMu0138	Polyribonucleotide nucleotidyltransferase, alpha chain (EC 2.7.7.8) (<i>pnpA</i>)	5.2/80 055	5.20 ± 0.00/77 000 ± 400	371 ± 57	1 157 ± 215
SMu0138	Polyribonucleotide nucleotidyltransferase, alpha chain (EC 2.7.7.8) (<i>pnpA</i>)	5.2/80 055	5.20 ± 0.00/77 200 ± 480	261 ± 132	1 535 ± 64
Translation					
Aminoacyl tRNA synthases					
SMu1441	Threonyl-tRNA synthase 1 (EC 6.1.1.3) (<i>thrS</i>)	5.4/74 758	5.33 ± 0.03/73 280 ± 790	41 ± 41	741 ± 81
SMu1441	Threonyl-tRNA synthase 1 (EC 6.1.1.3) (<i>thrS</i>)	5.4/74 758	5.40 ± 0.00/74 760 ± 0.0	101 ± 101	1 236 ± 384
SMu0592	Alanyl-tRNA synthase (EC 6.1.1.7) (<i>alaS</i>)	5.2/97 162	5.28 ± 0.02/88 230 ± 600	441 ± 147	1 283 ± 363
SMu1904	Arginyl-tRNA synthase (EC 6.1.1 NPT > 19), (<i>argS</i>)	6.0/63 574	6.10 ± 0.00/58 590 ± 580	1 715 ± 84	84 ± 84
SMu1374	Phenylalanyl-tRNA synthase, alpha subunit (EC 6.1.1.20) (<i>pheS</i>)	6.2/39 296	6.10 ± 0.00/40 200 ± 1 050	137 ± 137	6 936 ± 555
SMu1657	Glutamyl-tRNA (Gln) amidotransferase, B subunit (EC 2.6.-.-) (<i>gatB</i>)	5.1/53 501	5.08 ± 0.02/50 250 ± 280	604 ± 123	4 105 ± 127
SMu1658	Glutamyl-tRNA (Gln) amidotransferase, A subunit (<i>gatA</i>)	5.2/52 893	5.18 ± 0.03/52 960 ± 360	520 ± 41	2 233 ± 299

Table 1. cont.

Gene ID	Protein, EC no. (where applicable) and gene	2-DGE coordinates (pI/M _r)		DE value‡	
		Theoretical	Observed‡	pH 7.0	pH 5.0
Ribosomal proteins					
SMu1097	30S Ribosomal protein S1 (<i>rpsA</i>)	5.0/43 686	5.00 ± 0.00/50 170 ± 250	2 780 ± 72	13 569 ± 233
SMu1097	30S Ribosomal protein S1 (<i>rpsA</i>)	5.0/43 686	4.90 ± 0.00/50 250 ± 300	865 ± 381	7 463 ± 2 305
SMu1847	30S Ribosomal protein S2 (<i>rpsB</i>)	5.0/29 093	5.00 ± 0.00/33 150 ± 110	412 ± 75	2 240 ± 333
SMu1847	30S Ribosomal protein S2 (<i>rpsB</i>)	5.0/29 093	5.10 ± 0.00/33 050 ± 50	1 772 ± 88	8 036 ± 644
SMu1836	30S Ribosomal protein S3 (<i>rpsC</i>)	10.0/24 445	10.02 ± 0.20/27 300 ± 2 900	1 593 ± 68	6 411 ± 233
SMu1836	30S Ribosomal protein S3 (<i>rpsC</i>)	10.0/24 445	10.00 ± 0.36/27 610 ± 1 780	241 ± 121	3 990 ± 424
SMu1480	50S Ribosomal protein L1 (<i>rplA</i>)	9.4/26 674	9.35 ± 0.26/29 770 ± 2 070	1 415 ± 256	5 528 ± 591
SMu1480	50S Ribosomal protein L1 (<i>rplA</i>)	9.4/26 674	9.55 ± 0.15/28 190 ± 1850	2 188 ± 91	7 812 ± 833
SMu1480	50S Ribosomal protein L1 (<i>rplA</i>)	9.4/26 674	9.25 ± 0.03/30 050 ± 2 040	483 ± 173	2 003 ± 247
SMu0872	50S Ribosomal protein L7/L12 (<i>rplL</i>)	4.5/12 413	4.48 ± 0.02/9 580 ± 130	8 173 ± 5016	76 144 ± 7464
Translation elongation factors					
SMu0324	Elongation factor G (EF-G) (<i>fusA</i>)	4.8/76 664	4.80 ± 0.00/76 660 ± 0.0	1 141 ± 389	8 076 ± 1212
SMu0324	Elongation factor G (EF-G) (<i>fusA</i>)	4.8/76 664	4.80 ± 0.00/76 510 ± 0.0	4402 ± 1 045	12 935 ± 2204
SMu0324	Elongation factor G (EF-G) (<i>fusA</i>)*	4.8/76 664	5.00 ± 0.00/25 170 ± 40	925 ± 92	45 ± 17
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9/43 919	4.88 ± 0.04/56 820 ± 0.0	1 460 ± 204	10 156 ± 972
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9/43 919	4.88 ± 0.04/56 520 ± 0.0	0.0	11 115 ± 1751
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9/43 919	4.91 ± 0.04/57 000\$	20729 ± 10 281	98 340 ± 4 379
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9/43 919	4.98 ± 0.04/57 410 ± 440	1 145 ± 707	5 303 ± 1 400
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)	4.9/43 919	4.98 ± 0.02/52 630 ± 320	5 189 ± 415	820 ± 169
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)*	4.9/43 919	4.80 ± 0.00/26 640 ± 200	3 827 ± 48	1 825 ± 161
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)*	4.9/43 919	5.00 ± 0.00/22 340 ± 40	3 017 ± 172	618 ± 73
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)*	4.9/43 919	5.10 ± 0.02/20 300 ± 70	2 280 ± 204	572 ± 76
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)*	4.9/43 919	5.36 ± 0.31/20 360 ± 234	2 625 ± 331	476 ± 55
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)†	4.9/43 919	4.45 ± 0.05/13 720 ± 170	4 353 ± 331	830 ± 52
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)†	4.9/43 919	4.54 ± 0.05/12 960 ± 130	1 531 ± 49	57 ± 67
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)†	4.9/43 919	5.00 ± 0.00/25 170 ± 330	3 868 ± 296	1 240 ± 177
SMu0651	Elongation factor Tu (EF-Tu) (<i>tuf</i>)†	4.9/43 919	5.30 ± 0.00/22 260 ± 190	3 247 ± 508	1 099 ± 76
Protein conformation					
Molecular chaperones					
SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6/65 286	4.60 ± 0.00/64 840 ± 620	1 204 ± 172	5 529 ± 1 165
SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6/65 286	4.60 ± 0.00/65 180 ± 90	576 ± 299	6 277 ± 1 936
SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6/65 286	4.60 ± 0.00/65 290 ± 0.0	5 673 ± 248	28 513 ± 3 337
SMu1774	60 kDa Chaperonin (<i>groEL</i>)	4.7/57 101	4.70 ± 0.00/58 090 ± 240	535 ± 168	10 692 ± 844
SMu1774	60 kDa Chaperonin (<i>groEL</i>)	4.7/57 101	4.70 ± 0.00/57 380 ± 220	6 405 ± 344	44 202 ± 4 753
SMu1774	60 kDa Chaperonin (<i>groEL</i>)	4.7/57 101	4.70 ± 0.00/57 100 ± 110	1 217 ± 251	8 576 ± 2 025

Table 1. cont.

Gene ID	Protein, EC no. (where applicable) and gene	2-DGE coordinates (pI/M _r)		DE value‡	
		Theoretical	Observed‡	pH 7.0	pH 5.0
SMu0082	Peptidyl-prolyl isomerase (trigger factor; <i>ropA</i>)	4.5/47 486	4.51 ± 0.02/54 560 ± 400	6 653 ± 623	16 183 ± 1 705
Degradation of proteins and peptides					
SMu0587	Oligoendopeptidase B (EC 3.4.24.15) (<i>pepB</i>)	5.0/69 724	4.80 ± 0.00/43 090 ± 0.0	5 517 ± 585	1 324 ± 113
SMu0868	ATP-dependent Clp protease, ATP-binding subunit (EC 3.4.21.-) (<i>ClpL</i>)	5.0/77 186	4.98 ± 0.05/71 010 ± 50	0.0	3 492 ± 379
SMu1190	Dipeptidase (EC 3.4.13.18) (<i>pepD</i>)	5.0/53 037	5.06 ± 0.03/52 120 ± 310	135 ± 9	1 665 ± 191

*C-terminus of protein.

†N-terminus of protein.

‡Mean ± SEM (n=3).

§Part of the complement of random landmark proteins used to align gels and which were used for the calculation of the observed pI and M_r in accordance with the requirement of the z3 software.

in cells grown at neutral pH when subjected to a rapid drop in pH, but that cells grown at pH 5.0 can diminish the sensitizing effects of RecA deficiency (Quivey *et al.*, 1995). This physiological observation is a consequence of the induction, in *S. mutans* during growth at pH 5.0, of an abasic site-specific endonuclease activity which apparently acts independently of the RecA protein (Hahn *et al.*, 1999; Quivey *et al.*, 2001) in a similar manner to an error-prone form of DNA polymerase I (PolA) during the SOS response in *E. coli* (Lackey *et al.*, 1985; Wandt *et al.*, 1997). Even though a DNA polymerase I protein spot was identified in the 2-DGE proteome of *S. mutans*, it represented only the C-terminus of the protein, and was absent at pH 5.0 (Table 1).

Along with the enhanced expression of RecA, one of the two single-stranded DNA-binding proteins coded in the *S. mutans* genome, Ssb (Ajdić *et al.*, 2002), was uniquely expressed by growth of *S. mutans* at pH 5.0 (Fig. 1, Table 1). Both RecA and Ssb are essential for homologous genetic recombination as well as recombinational rescue and DNA repair of chromosomal replication. To the best of our knowledge, the only studies of this protein in streptococci relate to *S. pneumoniae* (Steffen *et al.*, 2002; Katz & Bryant, 2003), making this the first report of a role for Ssb in the acid tolerance of *S. mutans*. From the different roles for recombinational repair and the SOS response currently recognized in Gram-negative and Gram-positive bacteria, and the lack of information available on streptococci, it is clear that further research is needed to understand these processes in the acid-tolerant growth of *S. mutans*.

RNA synthesis and degradation

Under imposed stress conditions, it is self-evident that the DNA repair and protection responses needed for the survival of the cell require the transcription of appropriate genes. While the nature of these events has been well documented in *E. coli*, little is known of the mechanism(s) coordinating these events in streptococci, particularly oral streptococci (Volkert & Landini, 2001). Acid tolerance in *S. mutans*, however, led to upregulation of the transcription proteins, DNA-directed RNA polymerase α subunit, RpoA, and two isoforms of the transcription elongation factor (cleavage stimulatory factor) GreA, by 5.4-, 7.5- and 5.1-fold, respectively (Fig. 1, Table 1). In addition, one isoform of GreA was found to be uniquely expressed in the extracellular milieu at pH 5.0 (Fig. 1, Table 2). RNA polymerase forms an elongation complex with its template DNA and the nascent RNA product. While this complex is completely processive, it is responsive both to extrinsic regulatory factors and to intrinsic signals associated with the DNA and RNA that can alter the rate of elongation and lead to a transient pause or arrest of the complex (Nakasone *et al.*, 1998; Erie, 2002). This may be as simple as a lack of nucleotide substrates: a situation that may readily occur in *S. mutans* under acidic conditions, due to the disturbance of anabolic functions (Len *et al.*, 2004). The main function of GreA is to reactivate RNA polymerase once such a halt has occurred. This is achieved by enhancing the intrinsic

Table 2. Differentially expressed extracellular stress-related proteins from *S. mutans* grown at pH 7.0 or pH 5.0

Gene ID	Identification (gene)	2-DGE coordinates (pI/M _r)		DE value*	
		Theoretical	Observed*	pH 7.0	pH 5.0
Replication					
DNA replication and repair					
SMu1693	Single-stranded DNA-binding protein (<i>ssb</i>)	5.0/18 332	5.00 ± 0.00/17 120 ± 5	401 ± 95	18 ± 4
Transcription					
Transcription factors					
SMu1574	Transcription elongation factor (<i>greA</i>)	4.8/17 586	4.95 ± 0.04/18 640 ± 1 680	0.0	196 ± 43
Translation					
Translation factors					
SMu1846	Elongation factor Ts (<i>eftS</i>)	4.9/37 719	4.92 ± 0.04/41 650 ± 530	1 594 ± 644	12 ± 10
Ribosomal proteins					
SMu1097	30S Ribosomal protein S1 (<i>rpsA</i>)	5.0/43 686	4.88 ± 0.06/47 100 ± 500	1 162 ± 196	0.0
SMu0869	50S Ribosomal protein L10 (<i>rplJ</i>)	4.9/17 667	4.70 ± 0.00/14 960 ± 100	930 ± 1 030	11 ± 1
SMu0869	50S Ribosomal protein L10 (<i>rplJ</i>)	4.9/17 667	4.80 ± 0.00/10 300 ± 90	2 137 ± 273	81 ± 1
Cellular processes					
Molecular chaperones					
SMu0074	Chaperone protein DnaK (<i>dnaK</i>)	4.6/65 286	4.60 ± 0.00/65 290 ± 0	2 882 ± 739	85 ± 6
SMu0082	Peptidyl-prolyl isomerase (trigger factor; <i>ropA</i>)	4.5/47 486	4.47 ± 0.02/56 130 ± 340	1 787 ± 98	6 ± 6

*Mean ± SEM (n=3).

cleavage activity of RNA polymerase, thus releasing RNA from the elongation complex, and preventing backward translocation and hydrolysis of the RNA (Fish & Kane, 2002; Opalka *et al.*, 2003). Other than in the current study, elevated levels of GreA have been noted in the Gram-positive bacterium *Staphylococcus aureus*, when challenged with oxacillin (Singh *et al.*, 2001).

Two isoforms of the RNA-degrading enzyme polynucleotide phosphorylase [polyribonucleotide nucleotidyltransferase (PNPase; PnpA)] were also upregulated by at least 3.1-fold in *S. mutans* in acid-tolerant growth. (Fig. 1, Table 1). In *E. coli*, two 3'–5' exonucleases, RNase II and PNPase, are involved in mRNA decay, since the loss of both activities results in the cessation of growth (Donovan & Kushner, 1986). Recent analysis of RNA decay in *E. coli* (Mohanty & Kushner, 2003) has shown that even though RNase II constitutes about 90 % of the exonucleolytic activity in the cell (Deutscher & Reuven, 1991), PNPase plays a greater role in the degradation of mRNA, since it forms part of a multi-protein complex, the 'degradosome', that contains an RhlB RNA helicase capable of removing secondary structure that would impede PNPase activity (Mohanty & Kushner, 2003).

While the level of understanding of RNA decay in Gram-positive bacteria is not as advanced as that of Gram-negative bacteria, *in vitro* RNA decay does not appear to be as severely compromised by the absence of a PNPase activity (Wang & Bechhofer, 1996). In *Bacillus subtilis*, however, a *pnpA* deletion mutant shows pleiotrophic effects, including a

cold-sensitive-growth phenotype, sensitivity to growth in the presence of tetracycline and multiseptate, filamentous growth (Wang & Bechhofer, 1996). It has been hypothesized that defective processing of specific RNAs in the *pnpA* mutant of *B. subtilis* results in these phenotypes, though there is no direct evidence in support of this contention (Wang & Bechhofer, 1996). To date, no studies of the role of PNPase in oral streptococci appear to have been undertaken, though a 1.9-fold down-regulation of the enzyme has been observed in *Streptococcus oralis* when inoculated and grown in batch culture under aerobic conditions at low pH (Wilkins *et al.*, 2001). Our observation of an increase in PNPase is clearly at odds with this observation. Whether the aerobic conditions used to cultivate *S. oralis* engender an additional oxidative stress that further influences PNPase expression requires investigation.

Translation

The incorporation of correctly encoded amino acids into proteins depends on the attachment of each amino acid to an appropriate tRNA by aminoacyl tRNA synthases (Cooper, 2000). Acid tolerance in *S. mutans* resulted in the upregulation of phenylalanyl- (PheS), alanyl- (AlaS) and two isoforms of threonyl- (ThsS) tRNA synthases, as well as subunits A (GatA) and B (GatB) of glutamyl-tRNA amidotransferase, which is required for the transamidation of misacylated Glu-tRNA^{Gln} to form Gln-tRNA^{Gln} in all Gram-positive bacteria (Curnow *et al.*, 1997; Harpel *et al.*, 2002; Table 1). In contrast, arginyl-tRNA synthase (ArgS)

was down-regulated 20-fold by growth at pH 5.0 (Table 1). The other four aminoacyl tRNA synthases previously identified (Len *et al.*, 2003) did not show any differential expression at pH 5.0 (data not shown).

In *S. mutans*, the 50S and 30S ribosomal subunits are composed of 51 proteins (Ajdić *et al.*, 2002). Of these, five were identified as being upregulated by growth at pH 5.0, four of which existed in more than one charged isogenic form and possessed an observed M_r higher than that predicted from their gene sequence (Table 1). While 16 ribosomal proteins have previously been mapped on 2-DGE gels (Len *et al.*, 2003), the inability to detect all 51 proteins is most likely due to their low M_r and/or very basic pI (>10.5), which place them at the limit of the resolving power of current 2-DGE technology. Our data imply that the number of ribosomes may increase by a factor of four in a low-pH environment – a suggestion that will require independent confirmation. Protein S1 and the ribosomal protein L10 were also found in the culture medium, but at significant levels at pH 7.0 only (Table 2).

In translation, three elongation factors, EF-Tu, EF-Ts and EF-G, are responsible for escorting aminoacyl tRNAs to the ribosome and for translocation of the ribosome along the mRNA (Fig. 1; Cooper, 2000). A fourfold increase in the expression of the four charged isogenic forms of EF-Tu was observed by growing *S. mutans* at pH 5.0. In each case, the observed M_r of the protein was greater than that predicted from the gene sequence (Table 1). Two charged isogenic forms of EF-G were also upregulated at pH 5.0. Most notable, however, was the finding that the reduced amounts of multiple truncated forms of EF-Tu and a C-terminal fragment of EF-G in cells grown at pH 5.0 could be measured at all, as this meant that each protein spot had the same 2-DGE coordinates, irrespective of the growth pH (Table 1). This suggested that specific cleavage events had occurred, rather than random proteolysis. A similar observation has been made with *Salmonella enterica* serovar Typhimurium, where two specific proteinases are believed to be responsible for forming elongation factor artefacts (Adams *et al.*, 1999). Even if this were the case with *S. mutans*, the data in Table 1 indicate that the cumulative level of both EF-Tu and EF-G would be at least threefold higher during acid-tolerant growth at pH 5.0. Interestingly, no change in the third elongation factor, EF-Ts, was observed, except in the extracellular milieu, where its level was substantially upregulated at pH 7.0 (Fig. 1, Table 2).

The increase in EF-Tu and EF-G during acid-tolerant growth is also of interest from another standpoint, since it has recently been shown that both proteins behave like chaperones towards unfolded and denatured proteins in *E. coli* (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998, 2000). EF-Tu, for example, recognizes the same hydrophobic binding motifs in proteins as the chaperone DnaK (see below; Malki *et al.*, 2002). Furthermore, EF-Ts has been shown to act as a folding template in a chaperone-like manner towards its substrate protein, EF-Tu (Krab *et al.*, 2001).

Molecular chaperones and degradation of misfolded proteins

Early pulse-chase experiments demonstrated an increase in DnaK levels during thermal stress in *S. mutans*, thus confirming the existence of a functional heat-shock response-system in this species (Jayaraman & Burne, 1995). The DnaK chaperone machinery prevents the misfolding and aggregation of ribosome-bound polypeptides (Szabo *et al.*, 1994; Rudiger *et al.*, 1997; Bukau & Horwich, 1998; Agashe & Hartl, 2000). In the current study, proteome analysis identified three isoforms of DnaK in *S. mutans*, which were upregulated 4.6-, 10.9- and 5.0-fold, respectively, at pH 5.0 (Fig. 2, Table 1). This confirmed a similar observation made by Jayaraman *et al.* (1997), who used the same values of D and pH to culture *S. mutans*.

It is now clear that the function of DnaK overlaps with that of another component, trigger factor, RopA (Deuerling *et al.*, 1999; Teter *et al.*, 1999). RopA is a major ATP-independent molecular chaperone with prolyl isomerase activity, which binds to the large ribosomal subunit proteins L23 and L29 near the polypeptide exit site and interacts with nascent polypeptide chains prior to DnaK (Hesterkamp *et al.*, 1996; Kramer *et al.*, 2002). A 2.4-fold increase in RopA during steady-state growth at pH 5.0 was observed in *S. mutans* (Fig. 2, Table 1). Other molecular chaperones, such as the bacterial group I chaperonin, GroEL, are indispensable for cell viability (Fayet *et al.*, 1989; Kubota *et al.*, 1995). GroEL is able to capture and refold non-native substrate proteins up to 50–60 kDa, while protecting them from aggregation with other non-native proteins (Braig *et al.*, 1994; Mayhew *et al.*, 1996; Weissman *et al.*, 1996; Xu *et al.*, 1997). Proteome analysis showed the existence of three isoforms of GroEL in *S. mutans*, which were enhanced 20.0-, 6.9- and 7.0-fold, respectively, by growth in an acidic environment (Fig. 2, Table 1). While similar results have been reported for a 60 kDa chaperonin (most probably GroEL) in aerobic batch cultures of *S. mutans* and *S. oralis* (Wilkins *et al.*, 2001, 2002), the current results differed from those previously reported in continuous culture (Lemos *et al.*, 2001). In the previous study, *S. mutans* *groEL* mRNA was induced 2.5-fold by an acid shock from pH 7.0 to pH 5.0, but no significant differences in the levels of *groEL* mRNA or GroEL (determined by Western blot analysis) were observed, once steady state was achieved at $D=0.1\text{ h}^{-1}$ (Lemos *et al.*, 2001).

DnaK and RopA were also identified in the *S. mutans* culture fluid. Both proteins were down-regulated, by factors of 34 and 313, respectively, at pH 5.0 (Fig. 2, Table 2). These levels of DnaK and RopA were equivalent to 39 % and 36 %, respectively, of the steady-state cellular levels at pH 7.0, but were equivalent to less than 0.2 % of the steady-state cellular levels at pH 5.0, implying a loss (or secretion) rate of approximately $4.0\text{ \% h}^{-1}$ of the steady-state cellular levels at pH 7.0, but a negligible rate of loss at pH 5.0 (Jacques *et al.*, 1985). Although previous studies have not considered the extracellular milieu as a source of these proteins

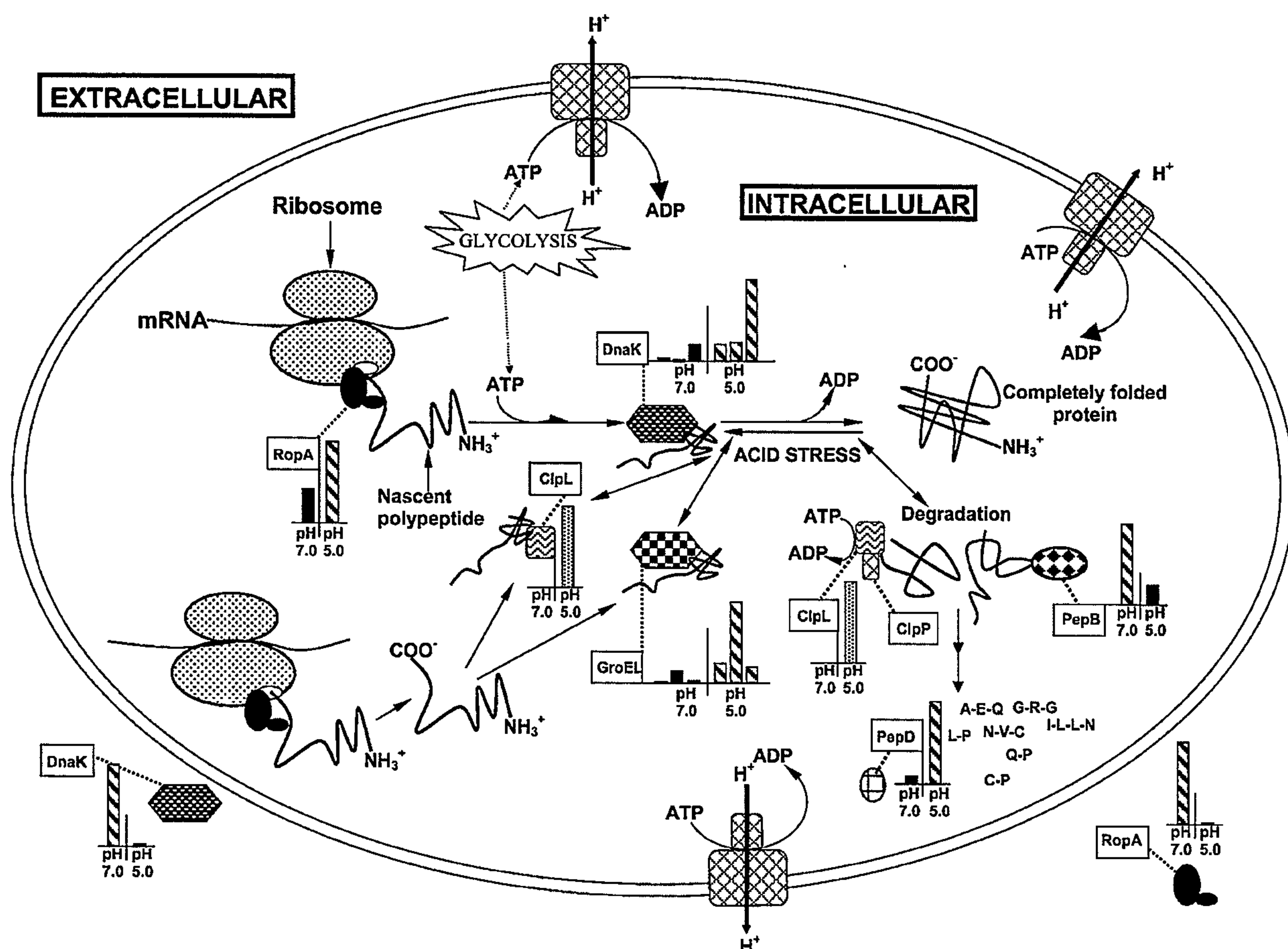


Fig. 2. Differentially expressed *S. mutans* molecular chaperones and proteinases from cells grown at pH 7.0 or at pH 5.0. The columns represent the percentage mean DE values for each charged isoform identified on 2-DGE gels, relative to the most highly expressed isoform in a given compartment. Protein spots were either upregulated (cross-hatching), down-regulated (black), or uniquely expressed (dots), relative to the alternative pH. Truncated forms of proteins are not shown, except for those observed in the extracellular milieu.

(Lemos *et al.*, 2001; Wilkins *et al.*, 2001, 2002), DnaK has recently been identified on the surface of both *Streptococcus agalactiae* (Hughes *et al.*, 2002) and *Haemophilus influenzae* (Hartmann *et al.*, 2001), and is known to be highly immunogenic in *S. pneumoniae* (Hamel *et al.*, 1997). Whether *S. mutans* has surface-bound molecular chaperones is a matter of conjecture, as our technique of protein extraction did not discriminate between the various cellular compartments. Irrespective of this, the increase in the steady-state cellular levels of DnaK, RopA and GroEL in *S. mutans* at pH 5.0 is consistent with a need for an enhanced complement of molecular chaperones to counteract the denaturing properties of an acidic cytosol when cytosolic pH falls below 6.5 (Dashper & Reynolds, 1992).

Proteins which cannot be folded by molecular chaperones may be targeted for degradation, in order to recycle amino acids for *de novo* protein synthesis (Jenal & Hengge-Aronis,

2003). Among the proteins that can carry out such functions are the ATP-dependent proteases of the Clp family, which possess a dual chaperone/proteinase role. Proteolysis by Clp requires a serine type peptidase, the ClpP subunit, and a regulatory ATPase subunit consisting of several orthologues (Gottesman *et al.*, 1997; Porankiewicz *et al.*, 1999; Lemos & Burne, 2002). In *S. mutans*, only one ATP-binding subunit, ClpL, was expressed, and solely in growth at pH 5.0 (Fig. 2, Table 1). ClpL homologues appear to be specific to Gram-positive bacteria, as they have not been found in Gram-negative bacteria (Lemos & Burne, 2002; Kwon *et al.*, 2003). To date, information on the role of ClpL is limited to a single study of the heat-shock response in *S. pneumoniae*, in which mutations in the *clpL* and *clpP* genes were found to modulate virulence-gene expression, and purified recombinant ClpL was shown to possess molecular chaperone properties (Kwon *et al.*, 2003). Our data suggest that ClpL is upregulated and maintained under low pH conditions, with

the implication that it plays a vital role in pH tolerance. It remains to be seen whether ClpL operates solely as a molecular chaperone, or whether it interacts with ClpP to initiate proteolysis.

Two other differentially expressed proteinases were identified by MALDI-TOF, both belonging to the *pep* gene family of proteinases, for which 13 different genes exist in the *S. mutans* genome (Ajdić *et al.*, 2002). One of these proteinases, a truncated version of a putative PepB, was down-regulated by a factor of 4.2 in growth at pH 5.0 (Fig. 2, Table 1). In Group B streptococci, PepB has oligopeptidase activity and has been shown to degrade a variety of small bioactive peptides, as well as the synthetic collagen-like substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala *in vitro* (Lin *et al.*, 1996). The second proteinase was homologous to the dipeptidase PepD of *Lactobacillus helveticus* (Ajdić *et al.*, 2002). This enzyme does not appear to be similar to the cytoplasmic PepD isolated from *S. mutans* and *S. sanguis* which catalyses the hydrolysis of X-Pro dipeptides (Cowman & Baron, 1997), since the *Lactobacillus* enzyme is not able to hydrolyse di- and tripeptides containing proline (Vesanto *et al.*, 1996). PepD was upregulated 12.3-fold at pH 5.0 (Fig. 2, Table 1).

It is perhaps pertinent to note that proteolysis has another role additional to the recycling of amino acids for *de novo* protein synthesis, and that is to regulate cellular events by degrading regulatory proteins, thereby controlling the response of the cell to an imposed stress. A readjustment in the composition of the cellular proteolytic machinery would therefore most likely have pleiotropic consequences for the cell, as any change in the nature or complement of specific proteinases would be expected to affect proteins that are subject to regulation by proteolytic events (Jenal & Hengge-Aronis, 2003).

Concluding remarks

This study has made use of the steady-state conditions enabled by anaerobic continuous culture in a chemostat to study alterations in the stress-response proteome of *S. mutans*, following adaptation and tolerance to growth at low pH. The proteome literature relating to acid adaptation and tolerance in oral streptococci contains disparate findings. For instance, proteome analysis of *S. mutans* during acid adaptation, using an aerobic batch-culture model without pH control, identified eight of the 28 differentially expressed proteins found in the current study. The levels of six of these were down-regulated by an average of 2.6-fold, while DNA-directed RNA polymerase was down-regulated 33-fold. Only a 60-kDa chaperonin was upregulated to a similar extent to that observed with GroEL in the current study. It is difficult to assess whether the aerobic conditions, the use of a different culture medium and/or the dissimilar generation times of 1.0 h and 6.6 h, respectively, for the control and the experimental batch cultures influenced the outcome (Wilkins *et al.*, 2002). Since an arbitrary ratio of the levels of expression has been evaluated in both studies, it may be that,

in batch culture, the control bacteria are adapting to a fall in the extracellular pH from a starting value of 7.0 to 6.2 at harvest (heading for a final of pH 5.3 at stationary phase) by initially overexpressing stress-related proteins. This would contrast with the steady-state levels measured in a chemostat, once *S. mutans* had adapted to the prevailing pH conditions. Such an overshoot in enzyme levels as an initial response to change has been noted previously, albeit in continuous culture (Carlsson & Elander, 1973; Koplove & Cooney, 1978), and if occurring in batch culture would explain the apparent reduction in the ratio between control and experimental protein values observed. Whatever the reason for the disparity, one must conclude that the two models are reflecting dissimilar events.

Although a number of stress-related proteins, in both Gram-positive and Gram-negative bacteria, are either well characterized or the subject of concerted ongoing study, others are not. This study has shown the involvement of at least three of these proteins in the acid-tolerant growth of *S. mutans*: the transcription elongation factor, GreA; the ATPase protease, ClpL; and the single-stranded DNA-binding protein, Ssb. The role of each of these proteins warrants further examination in light of the paucity of information regarding their mode of action. The subtle differences in homologous recombinational repair recently observed between *S. pneumoniae* and *E. coli*, along with the apparent differences in the complement of genes associated with the SOS-induced response in Gram-positive and Gram-negative bacteria (Steffen *et al.*, 2002; Katz & Bryant, 2003), emphasize this point, particularly as the regulons involved do not appear to have been studied in relation to acid tolerance or other stress responses in oral streptococci such as *S. mutans*.

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A proteomic approach to understanding the biofilm phenotype

The current theory for dental decay, the *ecological plaque hypothesis*, states that the onset of disease follows unfavourable disruption of the dynamic balance between the host and the microbial biofilm community at local sites¹. Inherent in this theory is the concept that cariogenic bacteria, such as *Streptococcus mutans*, can be present in health, but at levels that are not clinically relevant and that disease can be controlled not only by targeting pathogens but also by interfering with the factors responsible for driving the deleterious shifts. Such theories require an holistic or Systems Microbiological approach to study disease, as they are not tractable by traditional approaches alone. A 2004 report by the American Academy of Microbiology (<http://www.asm.org/academy/>) emphasised the technical bottlenecks associated with the systems' approach, including the need to obtain meaningful quantitative proteome data.

The word 'proteome' originated in 1994 when the Australian, Marc Wilkins, coined the term to describe the set of all PROTEins expressed by a genOME². While genomics and transcriptomics provide basic information on DNA sequences, regulatory elements and gene expression, proteomics provides quantitative information on the total protein profile of a cell by allowing the level of protein expression to be evaluated and the presence of protein isoforms and post-translational modifications to be detected. Although different methods are available to evaluate a cell's proteome³, only one method of proteome analysis, two-dimensional gel electrophoresis (2-DE), has so far been reported in the oral microbial field. The 2-DE allows visualisation of hundreds of proteins at a time. It relies on isoelectric focusing in the first dimension to separate proteins

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according to their pI, followed by SDS-PAGE in the second dimension to separate proteins according to their M_r . By using mass-spectrometry to identify the proteins, reference maps can be produced that document the functional proteome⁴. In practice, comprehensive proteome analysis requires an annotated protein database constructed from the genome of the species under investigation.

While the characterisation of proteins observed under a range of *in vitro* growth conditions is essential to completely define the proteome, differential or comparative proteomics are more frequently used to establish changes in protein expression between different physiological conditions, such as changes occurring during the planktonic to biofilm transition⁵. Recently reported studies with oral bacteria invariably use this approach⁶. One of the major drawbacks with conventional 2-DE proteome analysis is the number of gels needed to obtain statistically accurate data. This can be overcome by the use of 2-DE fluorescence difference gel electrophoresis (DIGE) developed by GE Healthcare Biosciences⁷. DIGE is based on labelling proteins with one of three fluorescent dyes prior to 2-DE allowing multiplexing of up to 3 separate protein mixtures on the same 2-DE gel. One of the advantages of the DIGE methodology is that an internal standard can be created

by pooling an aliquot of all of the samples one wishes to compare and labelling it with one of the CyDye DIGE fluors. This internal standard can then be run on each gel along with individual samples. This means that every protein from all samples is represented in the internal standard and that the internal standard is present on all gels for comparison with any two experimental conditions (Figure 1).

Approximately 95% of a bacterial genome codes for protein products. However, despite recent developments in narrow range Immobilised-pH-Gradient (IPG) strips used in the first dimension of 2-DE, the theoretical resolving power of 2-DE is still only about 75% of the proteome. Proteins with extreme pI values, especially very basic proteins, as well as very low and high M_r proteins are not readily resolved by current technology. In practice, many other proteins are poorly represented using 2-DE. These include low abundance proteins and hydrophobic proteins, particularly intrinsic cytoplasmic membrane proteins⁴. These latter membrane proteins represent between 15-30% of the bacterial proteome and yet are either not detected or represent <1.0% of the proteins displayed on 2-DE gels, even though some membrane-associated proteins, lipoproteins and outer membrane proteins of Gram-negative bacteria can be identified⁶.

The inherent difficulty in detecting, identifying and quantifying integral membrane proteins, high M_r surface antigens and low abundance proteins can be overcome by the use of an alternative to 2-DE, known as MUlti-Dimensional Protein Identification Technology (MuDPIT) in conjunction with iTRAQ labelling technology. In MuDPIT, the total protein mixture is first hydrolysed with a proteinase and the peptides separated by liquid chromatography before being passed onto an ElectroSpray Ionisation

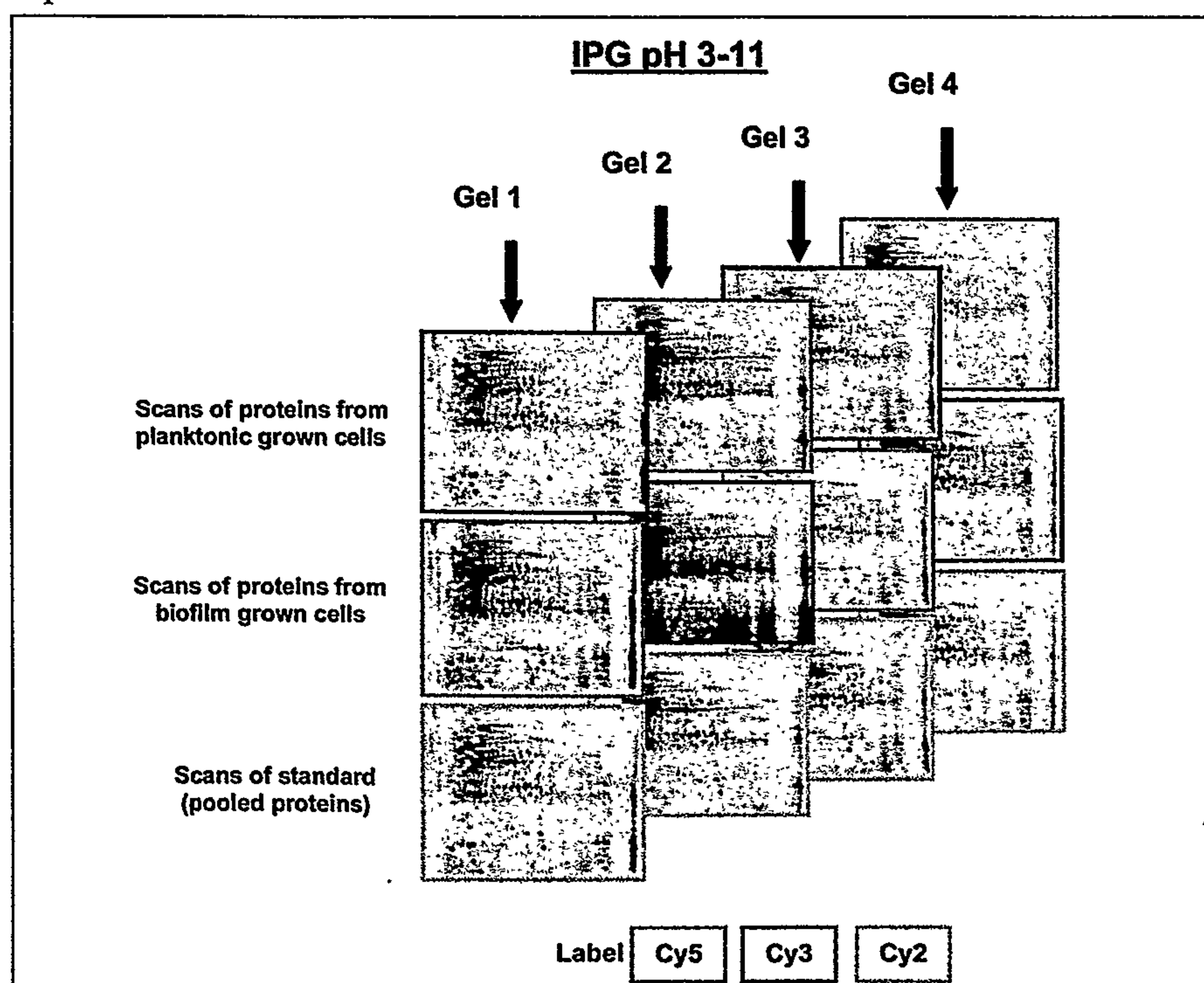


MS/MS mass spectrometer. This process allows the identification of the eluting peptides from their amino acid sequence which is determined by the mass spectrometer³. In late 2004, a method developed by Applied Biosystems for labelling peptides became available. Known as iTRAQ labelling technology, this technique enables peptides from different growth conditions to be labelled with an amine-specific tag. In a similar way to DIGE, this methodology allows both multiplexing of mixed samples as well as the absolute quantification of peptides from comparative experiments, something that was not possible with MuDPIT alone.

Over 65% of all microbial infections are due to bacterial biofilms rather than free living planktonic cells. The human pandemic oral diseases, such as dental caries, are no exception. So how does proteomics help us understand the physiology of bacteria in dental plaque on teeth? By using a proteomics approach, our studies at the Institute are allowing us to investigate the complexity of the phenotypic changes in the early colonising and protective bacterium, *Streptococcus gordonii* and the cariogenic pathogen, *S. mutans*. By comparing differences in the proteome that arise in planktonic and biofilm grown cells under a variety of imposed environmental conditions salient to the onset of disease, we are beginning to understand the complex pattern of changes that lead to the onset of virulence in *S. mutans* growing as a biofilm in dental plaque. These studies have already yielded novel findings resulting in the generation of a number of hypotheses that require further examination, particularly as some are unique or discordant with current dogma. One pertinent example is the role of stress-induced proteins in competence and biofilm formation. Research into competence is of considerable interest not only in the field of oral microbiology but also in other bacteria due to the involvement of gene transfer in virulence. Our hypothesis for a lack of any link in these processes in *S. mutans* biofilms⁵ represents a departure from the

emerging paradigm that this bacterium grows under stress in biofilms⁸. This notion is based primarily on cumulative results from isogenic mutants that do not take into account the natural phenotypic adaptive capacity of a bacterium with a full complement of genes. Proteomics overcomes this (inadvertently) imposed prejudicial constraint. Our recent adoption of DIGE and MuDPIT/iTRAQ techniques has allowed us to confirm and further extended our initial observations in a far more statistically accurate fashion, while the application of molecular techniques is allowing us to examine the uniqueness and relevance of the changes observed. Critical differences between the two streptococcal species determined by these combined approaches are providing the necessary impetus for a comprehensive strategy of intervention to be developed, thus allowing appropriate susceptible biochemical events to be targeted to eliminate disease.

Figure 1. 2-DE DIGE protocol and scans resulting from the Cy-Dye labelling procedure. In this instance each gel contains a mixture of a matched set of cellular proteins from steady state planktonic and biofilm grown cells with a standard composed of an equal aliquot pooled from each growth condition. Pooled standards are always labelled with Cy2 (orange outline) while the planktonic and biofilm sample are alternately labelled with either Cy3 (blue outline) or Cy5 (pink outline). Each gel is scanned at three different wavelengths, allowing the amount of a given protein to be measured and compared to the pooled standard. Comparison of the data across all four replicate gels allows for statistically significant changes in protein expression to be determined.



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